ACTIVATION OF ESTROGEN RECEPTOR ALPHA, ARYL HYDROCARBON RECEPTOR, AND NUCLEAR FACTOR ERYTHROID-2 LIKE 2 IN HUMAN BREAST CANCER CELLS

by

Raymond Ho Fai Lo

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmacology and Toxicology
University of Toronto

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Doctor of Philosophy

Department of Pharmacology and Toxicology
University of Toronto
2013

Abstract

There is a strong association between estrogen exposure and breast cancer risk. Estrogen can activate estrogen receptor α (ERα) to increase cell proliferation. Estrogen can also be metabolized into genotoxic compounds to induce DNA damage and mutations. Activation of the aryl hydrocarbon receptor (AHR) and nuclear factor erythroid-2 like 2 (NFE2L2; NRF2) can alter the production of genotoxic estrogen. The present thesis investigated the signalling mechanisms of ERα, AHR, and NRF2 and how their interaction might modulate breast cancer risk. In Chapter 2, genome-wide, but promoter-focused analysis of ERα binding sites in T-47D breast cancer cells identified potential cell line specific differences in estrogen signalling between T-47D and the commonly used MCF-7 breast cancer cells. CYP2B6 was identified to be an ERα target gene in T-47D cells but not MCF-7 cells, supporting cell line dependent effect in estrogen signalling. In Chapter 3 and 4, genome-wide analyses of AHR binding sites were performed to investigate the molecular criteria governing genomic AHR transactivation in vivo in mouse and in vitro in MCF-7 breast cancer cells. Our analysis identified 1) the previously established aryl hydrocarbon response element to be an important, but not an absolute
requirement in AHR transactivation and 2) key epigenetic modifications that modulate AHR-dependent gene regulation. Lastly, in Chapter 5, interaction among ERα, AHR, and NRF2 was presented at the regulatory region of two NRF2 target genes, NADPH Quinone Oxidoreductase 1 (NQO1) and Heme Oxygenase 1 (HMOX1). ERα repressed, whereas AHR enhanced NRF2-dependent NQO1 and HMOX1 mRNA expression through altered p300 recruitment and Histone H3 Lysine 9 acetylation. Collectively, this thesis examined novel molecular mechanisms that might alter breast cancer development/progression by modulating ERα, AHR, and NRF2 activity. (Word count: 278)
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<td>Gcl</td>
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Chapter 1
Introduction

1 Statement of Research Problem

Breast cancer is a complex and heterologous disease, modulated by many genetic and environmental factors. However, studies have consistently shown a strong correlation between breast cancer risk and the duration of estrogen exposure. For example, an increase in breast cancer risk has been observed for women who experience early menstruation and late menopause. To account for this correlation, there are two co-existing molecular pathways that might contribute to hormonal dependent breast cancer progression/development.

Estrogen is a mitogen and can induce cell proliferation and tumor progression. At the same time, estrogen can be metabolized to genotoxic metabolites, which initiate critical mutations in the genome to initiate tumor formation. Phase I and Phase II enzymes regulate the formation and elimination of these genotoxic metabolites. Interestingly, the estrogen receptor alpha (ERα), the aryl hydrocarbon receptor (AHR), and the nuclear factor erythroid-2 like factor 2 (NRF2) all play important roles in etiology of breast cancer, by modulating the Phase I and Phase II gene expression. This thesis aims to investigate the genomic activation of ERα, AHR, and NRF2 and how simultaneous activation of these transcription factors influences the expression of genes involved in the bioactivation and the detoxification pathways of breast cancer development.

ERα is a ligand-activated transcription factor that mediates the mitogenic effect of estrogen. While the genome-wide binding profiles of ERα have been reported in MCF-7 breast cancer cells, there have been no reports of ERα binding sites in other breast cancer models. Our comparison between the genomic binding sites of ERα in MCF-7 and T-47D breast cancer cells (outlined in Chapter 2) reveals that CYP2B6 is differentially regulated in the two cell lines, highlighting potential cell line differences in estrogen signaling. Furthermore, our study highlights that ERα might enhance xenobiotic metabolism in breast tissues by regulating CYP2B6 expression in addition to CYP1A1, CYP1B1, and CYP2A6. Enhanced xenobiotic metabolism might increase the production of reactive intermediates, thereby increasing the likelihood of tumor-initiating mutations.
Unlike ERα, AHR has traditionally been considered as a regulator of Phase I enzymes such as CYP1A1 and CYP1B1. Both CYP450s have been demonstrated to metabolized estrogens into genotoxic metabolites, hence an improved understanding of AHR signalling is needed. Genome-wide analysis of AHR binding sites in vivo has not been conducted. Moreover, no one has conducted a comprehensive, full genome-wide analysis of AHR binding sites in breast tissue. Our studies outlined in Chapter 3 and Chapter 4 identify genome-wide AHR binding sites in vivo and in vitro and highlight epigenetic events that are required for full AHR transactivation.

NRF2 is the regulator of Phase II enzymes involved in the detoxification and elimination of genotoxic estrogen metabolism, and hence has tremendous implications in the prevention of breast cancer. AHR and ERα modulate NRF2 transcriptional activity with AHR having a positive and ERα having an inhibitory effect. However, there have been no studies investigating how NRF2 activity might be affected by simultaneous activation ERα and AHR. Chapter 5 of this thesis examines the effect of 3,3’-diindolylmethane (DIM), a dual ERα and AHR activator, on NRF2 transcriptional activity and the regulation of Phase II detoxification pathways in breast cancer.
1.1 Estrogen Receptor

The physiological effects of estrogen are primarily mediated by two estrogen receptors – estrogen receptor alpha (ESR1, ERα, nuclear receptor subfamily 3 group A member 1) and estrogen receptor beta (ESR2, ERβ, nuclear receptor subfamily 3 group A member 2). They are both ligand-activated transcription factors that belong to the nuclear receptor superfamily (Germain et al., 2006; Heldring et al., 2007; Lubahn et al., 1993). ERα was first described by Dr. Elwood Jensen in 1962 (Jensen EV, 1962), purified, and cloned by Dr. Pierre Chambon (Green et al., 1986b; Greene et al., 1977). ERβ on the other hand, was first cloned and characterized in rat by Dr. Jan-Ake Gustafsson in 1996 (Kuiper et al., 1996). In the canonical genomic pathway of estrogen signalling, estrogenic compounds diffuse into the nucleus and bind ER. This results in receptor conformational change and homodimerization. The ER homodimers then interact with estrogen response elements (ERE) in the genome to mediate gene regulation.

1.1.1 Functional domains of ERα

The full length ERα gene (ESR1) is located on chromosome 6q25.1 (chr6:152,128,454-152,424,408) and comprises eight coding exons that together code for a polypeptide of 595 amino acids with a molecular weight of 66.2 kDa (Greene et al., 1986; Ponglikitmongkol et al., 1988). Similar to other members of the nuclear receptor superfamily, ERα can be divided into 6 functional domains of homology (Green et al., 1986a), which are labelled alphabetically (A-F) from the N-terminus to the C-terminus (Germain et al., 2006; Kumar et al., 1987). The N-terminal A/B region of ERα contains the activation function 1 (AF1) which can mediate ligand-independent gene transactivation. This is supported by the fact that a chimeric protein containing the DNA binding domain and the AF1 domain can confer low constitutive transactivation activity in the absence of estrogen (Lees et al., 1989). The AF1 domain also enhances and is required for maximal gene transactivation in a ligand-dependent manner in response to estrogen stimulation (Kumar et al., 1987; Tzukerman et al., 1994). Ligand-dependent and ligand-independent actions of the AF1 domain are likely conferred through post-translational modification such as serine phosphorylation. Estrogen binding to the receptor (Joel et al., 1995) or activation of the MAPK pathway (Bunone et al., 1996)
leads to significant phosphorylation at serine 104, 106, 118, and 154 in the AF1 domain, which facilitates interaction with transcriptional coactivators (Endoh et al., 1999), leading to enhanced ERα activity.

**Figure 1** Schematic mapping of the functional domains in estrogen receptor alpha.

ERα is divided into six domains. The functions associated with each domain are indicated by a line. AF1, Activation function 1; AF2, Activation function 2; DBD, DNA binding domain; ERα, estrogen receptor alpha; LBD, Ligand binding domain.

ERα homodimerization and ERα-DNA interaction are mediated by two cysteine-rich zinc finger motifs located in the C region of ERα. Each zinc finger consists of four cysteines organized in a tetrahedral formation, which forms an interaction pocket for the zinc ion (Ruff et al., 2000). DNA sequence recognition is mediated by the highly conserved P box (residues around the last two cysteines in the first zinc finger), whereas the D box (residues between the first two cysteines in the second zinc finger) determines spacing between the two ERα homodimers (Ruff et al., 2000). The D region, termed the hinge region, is a flexible region that contains nuclear localization signals and connects the DNA binding domain to the ligand binding domain (Germain et al., 2006; Ruff et al., 2000).

The C-terminal E/F region contains the ligand binding domain and is required for ligand-dependent transactivation (Kumar et al., 1987). The ligand binding domain (LBD) can be divided into four functional regions – 1) homodimerization surface, 2) ligand binding pocket, 3) coregulator binding surface, and 4) transactivation region. The ligand binding
pocket is formed by 11 α-helices (helices H1 – H12) and enclosed by a β-sheet. Ligand specificity is determined by H-bond interaction between estrogen and the polar residues in the ligand binding pocket. Crystal structure of the liganded estrogen receptor indicates that the 3-OH moiety of 17β-estradiol participates in H-bond interaction with residues R394 on helix H5 and E353 on helix H3 whereas the 17-OH moiety interacts with H524 on helix 11 (Brzozowski et al., 1997). Homodimerization is mediated by helices 8 – 11, linking ligand interaction and homodimerization events. Upon agonist binding, the hydrophobic regions of helix 3, 4, 5, and 12 adopt a conformation that facilitates interaction with coactivators containing the residue sequence LXXLL (Brzozowski et al., 1997; Shiau et al., 1998). Interaction with coactivators subsequently mediates gene regulation.

1.1.2 Estrogen signal transduction

1.1.2.1 17β-estradiol and other estrogenic compounds

Estrogens belong to a family of steroidal sex hormones and activate the estrogen receptor alpha and beta to regulate various physiological responses including development, sexual maturation, and reproduction. The most potent physiological estrogen in human is 17β-estradiol (E2), a four ringed steroidal structure hydroxylated at the C3 and C17 position with a molecular weight of 272.38 g/mol. E2 is primarily produced in the ovaries, whereas secondary sites of production are located in the adrenal cortex, testes, and adipocytes (Gruber et al., 2002). Through a series of oxidation and hydroxylation steps, cholesterol is converted to androstenedione and testosterone, whose A rings are aromatized by aromatase to form estrone (E1) and E2, respectively. The circulating level of E2 varies substantially depending on the gender, age, and menstrual phase. Serum E2 level is the highest in preovulatory women, reaching concentration as high as 500 pg/mL (calculated to be approximately 1.84 nM) and as low as <20 pg/mL (<73.6 pM) in post-menopausal women and men (Gruber et al., 2002). Interestingly, it has been suggested that local tissue concentration of E2 might be ten to forty times higher in post-menopausal breast tumours compared to circulating concentration (Miller et al., 1982). The other two less potent endogenous estrogens, estrone (E1) and estriol (E3), are produced predominantly in post-menopausal women and pregnant women respectively.
In addition to endogenous estrogens, some synthetic and phyrogenic compounds possess weak estrogenic activity and can affect hormonal homeostasis in the body. The hallmark of an estrogen ligand is the phenolic group on the A ring (Fang et al., 2001; Jordan et al., 1985). Ligands that mimic the action of estrogens usually contained a similar polar moiety. Known phytoestrogens include genistein, coumestrol, and, resveratrol, whereas known xenoestrogens include pharmaceutical compounds such as ethinylestradiol, tamoxifen, raloxifene, diethylstilbestrol, and equilin, which are indicated for various situations or conditions including oral contraception, menopause, breast cancer, and elevated pregnancy risk (Lewis and Jordan, 2005; Machado et al., 2011; Rasmussen, 2012). Xenoestrogens also include synthetic compounds used in the manufacturing and industrial sectors such as bisphenol A and polychlorinated biphenyl metabolites, pesticides such as DDT, and synthetic compounds used in laboratory settings such as 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), diarylpropionitrile (DPN), and tetrahydrochrysene (THC). Many compounds are still currently being screened for their estrogenicity in the US EPA endocrine disruptor screening program (Willett et al., 2011).
Figure 2 Chemical structures of various known estrogenic compounds Genomic pathway of estrogen signalling
1.1.2.2 Genomic pathway of estrogen signalling

The genomic pathway of estrogen signalling, also termed the classical pathway describes the mechanism by which estrogen activates the estrogen receptor, leading to receptor-DNA interaction and changes in target gene expression. In the absence of ligand, ERα resides in the nucleus coupled to chaperone proteins for stabilization. Ligand binding releases the receptor from its chaperone protein, inducing conformational changes and receptor homodimerization (Pearce and Jordan, 2004). The receptor dimer interacts with ERα binding sites that are located in both proximal promoter regions and distal cis-regulatory regions in the genome (Carroll et al., 2005; Carroll et al., 2006; Hurtado et al., 2011). ERα binding sites typically contain an estrogen response element (ERE), which is a 13 bp inverted palindromic sequence with a 3 bp spacer (5’AGGTCAnnnTGACCT3’). The ERE has been shown to be indispensable for the regulation of several known estrogen target genes including trefoil factor 1 and vitellogenin A2, although recent genome-wide analysis of ERα binding sites has concluded that the ERE is not a stringent requirement for ERα-DNA interaction (Carroll et al., 2005). ERα-DNA interaction can also be mediated through variations of the ERE (such as a half-site ERE with the sequence 5’AGGTCA3’) or through protein-protein tethering. For example, ERα has been demonstrated to interact with DNA indirectly by tethering to other transcription factors such as AP1 and Sp1 (Pearce and Jordan, 2004; Safe and Kim, 2008). Recent studies have shown that the criteria of ERα binding extend beyond simple DNA sequence recognition and involve the concerted cooperation among ERα, pioneer transcription factors, and chromatin modification. Pioneer factors such as CCCTC-binding factor (CTCF), Cohesin, Forkhead protein A1, and histone methylation might all play important roles in determining ERα-DNA interaction (Hurtado et al., 2011; Schmidt et al., 2010).
Figure 3 Genomic pathway of estrogen signalling.

E2 diffuses into the nucleus to activate and induce ERα dimerization. The ERα dimer can interact directly with ERE to form a pre-initiation complex to regulate gene expression. ERα can also interact with DNA indirectly by tethering to transcription factors such as AP-1 and SP1. DIM has been reported to activate ERα indirectly by inducing PKA and MAPK-dependent phosphorylation of ERα.

Estrogen receptors can also be activated independent of direct ligand interaction. 3,3'-diindolylmethane (DIM), for example, does not bind ER (Riby et al., 2000) but does activate ERα and ERβ through phosphorylation of the receptor and related co-regulatory proteins (Leong et al., 2004b; Vivar et al., 2010). Leong et al. have demonstrated that DIM induces phosphorylation of ERα and cAMP response element binding protein (CREB) to up-regulate an ERE reporter gene through MAP Kinase and Protein Kinase A dependent pathways (Leong et al., 2004b). DIM can also induce ERβ and Nuclear Coactivator 2
recruitment to the *Keratin 19* gene. The precise mechanism was not investigated, although Vivar *et al.* postulate that activation of ERβ is facilitated through protein phosphorylation similar to ERα (Vivar *et al.*, 2010). There is likely another class of ER activators that do not bind ER, but rather activate ER through phosphorylation. Given that different ligands of ER can induce differential receptor conformation changes and gene expression pattern, it is conceivable that ER activators like DIM might result in gene expression patterns that are distinct from E2.

### 1.1.2.3 Non-genomic pathway of estrogen signalling

The non-genomic pathway of estrogen signalling describes the mechanism by which estrogen induces cellular molecular changes independent of nuclear estrogen receptor. In comparison to the genomic pathway, the non-genomic pathway is considerably more rapid (within seconds to minutes) and hence might not involve altered transcription or protein synthesis (Nilsson *et al.*, 2011). The non-genomic action of estrogen is believed to be mediated through G-protein coupled receptor 30 (GPR30) and involves traditional players in GPCR signalling cascades such Gαs, adenylyl cyclase, cAMP, and Protein Kinase A (Nilsson *et al.*, 2011).

### 1.1.3 Physiological role of ERα

ERα is abundantly expressed in all female reproductive tissues and its physiological role in the sexual maturation is well-documented. ERα knockout (αERKO) animals are infertile due to reduced post-pubertal uterine growth, anovulatory and the disruption of luteinizing hormone secretion (Couse and Korach, 1999a, b; Lubahn *et al.*, 1993). Lack of ERα abrogates proper mammary gland development, likely through the disruption of estrogen signalling. Patients who lack the ability to produce endogenous estrogen do not develop mammary gland tissues at puberty, which can be corrected by the administration of estrogen (MacGillivray *et al.*, 1998). A clear correlation among estrogen exposure, ERα expression, and breast cancer development has been established in multiple epidemiological studies, further confirming the important role estrogen and ERα play in the breast tissue.
ERα also plays important physiological roles in the skeletal and cardiovascular tissues. The effect of estrogen on the skeletal system is best illustrated by the increased incidence of osteoporosis in post-menopausal women (MacGillivray et al., 1998). Estrogen maintains bone density by modulating bone growth and resorption. Female αERKO exhibits decreased bone mineral content and femur length (Couse and Korach, 1999a), while patients with compromised estrogen production exhibit similar decrease in bone mineral content, density, and maturation (MacGillivray et al., 1998).

Estrogen has been proposed to exert a protective effect on the cardiovascular system (Farhat et al., 1996) likely through its non-genomic signalling pathway (ie. independent of ERα). For example, estrogen interacts with surface membrane receptor, initiating a cascade of secondary messengers that leads to increased endothelial nitric oxide synthase and vasodilation (Chen et al., 1999). Clinically, the protective effect of estrogen replacement therapy on the cardiovascular system is debatable. In fact, the women’s health initiative (WHI), a large multi-centered, observational study investigating the effect of post-menopausal hormone replacement therapy, was suspended mid-trial due to an increase risk of coronary heart diseases (CHD) in the estrogen plus progestin arm (Rossouw et al., 2002), and an increase risk of strokes and pulmonary embolisms in the estrogen-alone arm (Hulley and Grady, 2004).

Estrogens are established mitogens and affect cell cycle and cell proliferation in many hormonal sensitive cell lines. The effect of estrogen on cell proliferation and tumour growth has been demonstrated in studies as early as 1896 when oophorectomy to decrease estrogen level has been the standard treatment for estrogen-dependent breast cancer (Stockwell, 1983). The mitogenic effect of estrogen is likely mediated through the induction of several pro-mitogenic genes including cyclin D1, c-myc, and c-fos (Lu and Serrero, 2001; Watson et al., 1991). The expression levels of cyclin D1, c-myc, and c-fos are estrogen- and ERα-dependent (Dubik et al., 1987; Foster and Wimalasena, 1996; Weisz and Bresciani, 1988). Cyclin D1 or c-myc expression can lead to the activation of cyclin E-cdk2, phosphorylation of retinoblastoma protein, and progression into S phase (Prall et al., 1998). Activated c-fos heterodimerizes with c-Jun to form the transcription factor complex AP-1, which regulates genes involved in cell proliferation (Shaulian and Karin, 2001).
Overexpression of c-fos can also stabilize cyclin D1 to accelerate cell cycle progression (Guller et al., 2008).

### 1.1.4 Estrogen receptor beta

In addition to ERα, ERβ also regulates various physiological processes in response to endogenous and exogenous estrogens. However, since ERβ is not expressed in any of the biological models that I have employed in this thesis, its physiological role and functional domains will only be described briefly in this section. ERβ is coded by a separate gene located on chromosome 14q23.2 (chr14: 64,699,747 - 64,761,128). The LBDs of the two receptors have 55% amino acid identity and bind to E2 with similar affinity (Kuiper et al., 1998). However, at 55% amino acid identity, the two LBDs are also distinct enough that they have different specificities for various estrogenic ligands (Kuiper et al., 1997; Sun et al., 2003). Similarity between the two estrogen receptors is the highest in the DNA binding domain with greater than 95% amino acid homology (Gustafsson, 1999). Similarity in the DNA binding domain suggests a high degree of overlap in their respective target genes, which has been verified by a recent ChIP-Seq study comparing the binding sites for both estrogen receptors in MCF-7 breast cancer cells that were engineered to express both receptors (Grober et al., 2011). Despite substantial overlap in their target genes, ERβ knockout (βERKO) mice have similar but also distinct phenotypes compared to their ERα counterparts. Female βERKO mice have significantly reduced fertility whereas male βERKO mice are fully fertile (Krege et al., 1998). Furthermore, lack of ERβ expression does not drastically affect mammary gland development, implying that ERα is the predominant receptor involved in the mammary gland development (Couse and Korach, 1999a). The role of ERβ in breast cancer remains unclear due to the expression of different ERβ splice variants in the mammary gland, but has been suggested to have an inhibitory effect on ERα when co-expressed (Matthews and Gustafsson, 2003; Pettersson et al., 2000). One example that might be relevant to breast cancer is their opposing effects on the expression of cyclin D1. ERα up-regulates whereas ERβ down-regulates the expression of cyclin D1, a gene involved in cell cycle progression (Liu et al., 2002).
1.2 Aryl Hydrocarbon Receptor

The existence of the aryl hydrocarbon receptor (AHR) was first proposed in 1970 by Dr. Dan Nebert (Nebert and Bausserman, 1970a), verified using radiolabelled dioxins by Dr. Alan Poland in 1976 (Poland et al., 1976), and finally cloned by Dr. Christopher A. Bradfield and Dr. Yoshiaki Fujii-kuriyama in 1992 (Burbach et al., 1992; Ema et al., 1992). The cloning of the AHR reveals that although AHR behaves similar to the many steroidal nuclear receptors, it belongs to a completely different family of transcriptional regulators. The AHR belongs to the class I basic helix-loop-helix (bHLH) PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM) (PAS) family of transcriptional regulators (Gu et al., 2000; Kewley et al., 2004), and is primarily responsible for mediating xenobiotic responses upon exposure to planar compounds including polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH) (Bock and Kohle, 2006). In the canonical genomic pathway of AHR signalling, AHR ligand such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) diffuses through the cell membrane and activates the AHR in the cytosol. AHR then translocates to the nucleus, dimerizes with the AHR nuclear translocator (ARNT), and binds to aryl hydrocarbon response elements (AHRE) in the genome to mediate gene regulation.

1.2.1 Functional domains of AHR

The AHR is located on chromosome 7p21.1 (chr7: 17,338,276-17,385,775) and comprises eleven coding exons that together code for a polypeptide of 848 amino acids with a molecular weight of 96 kDa (Dolwick et al., 1993). Similar to other members of the bHLH/PAS transcription factors, the AHR protein can be roughly divided into 4 functional domains of homology. The four domains are, from N-terminus to C-terminus, the bHLH (aa 27-79), PAS A (aa 121-182), PAS B (aa 259-374), and transactivation domains (Gu et al., 2000; Kewley et al., 2004). AHR interaction with TCDD is reduced by 30% in mutants lacking the PAS A domain and completely abolished in mutants lacking the PAS B domain (Fukunaga et al., 1995). Hence, the ligand binding pocket is located in the PAS domains. The crystal structure of AHR has yet to be determined. However, computational modeling of the AHR ligand binding pocket using information from another member of the bHLH PAS family suggests that the ligand binding pocket consists of a centrally located β-sheet flanked
by two helices and connected by a medial “belt” (Xing et al., 2012). The ligand binding pocket is lined with hydrophobic residues which maintain ligand-receptor interaction and ligand-induced receptor activity (Pandini et al., 2009). Ligand specificity for TCDD is conferred by the polar Gln377 (murine AHR) which interacts with the oxygen atom of TCDD via H-bond and Gly298 which interacts with the lateral position of TCDD (Xing et al., 2012).

![AHR schematic](image)

**Figure 4 Schematic mapping of the functions domains in the aryl hydrocarbon receptor.**

AHR can be roughly divided into four domains – bHLH, PAS A, PAS B, and TAD. The functions associated with each domain are indicated by a line. bHLH, basic helix-loop-helix; PAS, Per-ARNT-SIM; Hsp90, heat shock protein 90; TAD, transactivation domain.

The bHLH, PAS A and PAS B domains are all indispensable for 1) the dimerization of AHR and ARNT, and 2) AHR interaction with DNA (Fukunaga et al., 1995; Lindebro et al., 1995). The bHLH and the PAS domains preferentially interact with an atypical E-box (5’CANNTG3’) DNA sequence motif (Chapman-Smith and Whitelaw, 2006). Deletion of bHLH and PAS B domain reduces AHR interaction with a 90 kDa heat shock protein (Hsp90), AHR’s cytosolic chaperone protein (Fukunaga et al., 1995). Taken together, bHLH domain is responsible for dimerization with ARNT, interaction surface for Hsp90, and DNA
interaction. The PAS A and B domains are responsible for ligand interaction and ARNT dimerization.

The transcriptional activity of AHR is mediated by the C-terminal transactivation domain (TAD), where deletion of the C-terminus transactivation domain abolishes receptor activity without affecting ARNT dimerization and DNA interaction (Fukunaga et al., 1995). The TAD can be divided into three functional regions – acidic region, Q-rich region, and P/S/T region. Interestingly, deletion of the human AHR P/S/T region enhances transcriptional activity while a L678A residue substitution in the Q-rich region completely attenuates AHR transactivation by decreasing interaction with transcriptional co-activator (Kumar et al., 2001). On the other hand, a chimeric mouse AHR containing only the N-terminus and the acidic region possessed full activity, suggesting that the acidic region and the Q-rich region might be able to work independently to mediate gene transactivation (Jones and Whitlock, 2001). Regardless of the precise mechanism or the functional domains involved, it is clear that the C-terminal portion of AHR is responsible for gene transactivation.

1.2.2 AHR signal transduction

1.2.2.1 TCDD and AHR ligands

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can lead to hepatomegaly, thymic involution, cleft palate, chloracne, and wasting syndrome in TCDD-sensitive animals (Poland and Knutson, 1982). TCDD is the prototypical activator of AHR and represents a class of anthropogenic and/or environmental AHR ligands called halogenated aromatic hydrocarbons (HAHs). HAHs are planar ligands with high binding affinity to AHR and include persistent organic pollutants such as polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (Denison and Nagy, 2003). PCBs are industrial chemicals used primarily as flame retardants and electric fluids in capacitors, whereas PCDDs and PCDFs are by-products of incomplete combustion processes such as waste incineration. The manufacturing/import and the intentional release of PCBs into the environment were made illegal in 1977 and 1985 respectively due to PCBs deleterious effects (reproductive failures and birth defects in
wildlife) on the ecosystem and their potential to bioaccumulate in the food chain (Canada. Environment Canada., 1990). Environmental levels and human tissue levels of HAHs have dropped substantially since the installment of heavy government regulation; however, traces of HAHs can still be detected in the human population and environment. Although the mechanism remains unclear, ubiquitous HAH exposure has been associated with a wide range of epidemiological diseases including obesity (Verhulst et al., 2009), diabetes (Consonni et al., 2008; Kerkvliet et al., 2009; Uemura, 2012), pulmonary diseases and cancers (Consonni et al., 2008). In addition to HAHs, there exists another class of anthropogenic/environmental AHR ligands called polycyclic aromatic hydrocarbons (PAHs). PAHs include B[a]P, 3-methylchololanthrene (3MC), benzantracenes, and benzoflavones. B[a]P and benzantracenes are products of incomplete combustion whereas 3MC and 5,6-benzoflavone (also known as beta-naphthoflavone βNF) are synthetic laboratory compounds (Denison and Nagy, 2003).

AHR ligands are structurally diverse. In recent years, researchers have identified a growing list of naturally occurring (both physiological and dietary) AHR ligands. AHR plays important roles in a diverse range of physiological processes, and therefore it is reasonable to suggest the existence of an endogenous AHR ligand that might mediate those physiological processes. Several candidate endogenous AHR ligands have emerged, including tryptophan metabolites such as 6-formylindolo[3,2-b]carbazole (FICZ) (Wei et al., 2000; Wincent et al., 2012) and kynurenine (DiNatale et al., 2010; Opitz et al., 2011), and tetrpyroles from heme biodegradation such as bilirubin and biliverdin (Phelan et al., 1998; Sinal and Bend, 1997). Dietary compounds derived from vegetables, fruits, tea, and wine represent another class of AHR ligands. Cruciferous vegetables are rich in indole-3-carbinol (I3C), which is a weak AHR activator (Denison and Nagy, 2003). Under acidic condition in the GI tract, I3C is metabolized into potent AHR agonists including 3,3’-diindolylmethane (DIM, an ER activator described in Section 1.1.2.1) and indolo[3,2-b]carbazole (ICZ). Many dietary AHR ligands including resveratrol, kaempferol, quercetin, myricetin, and luteolin act as AHR antagonists whereas galangin, genistein, daidzein, diosmin, and cantharidin possess some AHR agonist activity (Zhang et al., 2003). Some of the ligands mentioned above are also
estrogen activators at micromolar concentrations, highlighting the overlapping ligand recognition between AHR and ER.

Halogenated Aromatic Hydrocarbons

![TCDD](image1), ![PCB 126](image2), ![TCDF](image3)

Polycyclic Aromatic Hydrocarbons

![BaP](image4), ![3MC](image5), ![beta-Naphthoflavone](image6)

Potential Endogenous AHR ligands

![FICZ](image7), ![Kynuerine](image8), ![Billirubin](image9)

Naturally occurring AHR ligands

![ICZ](image10), ![DIM](image11)

Figure 5 Chemical structures of various known AHR agonists.
1.2.2.2 Aryl hydrocarbon receptor translocator

The aryl hydrocarbon receptor translocator (ARNT; also known as hypoxia inducible factor 1 beta) belongs to the Class II bHLH PAS family of transcription factors and is the indispensable heterodimerization partner for many bHLH proteins including AHR, hypoxia-inducible factor 1 and 2 and single-minded protein 1 and 2. ARNT has been reported to enhance the transcriptional activity of ERα and ERβ (Brunnberg et al., 2003; Ruegg et al., 2008). ARNT enhances their transcriptional activity likely by enhancing the recruitment of and interacting with Nuclear Coactivator 1 and 2 through helix 2 in the bHLH domain and with CBP/p300 in the C-terminus transactivation domain (Beischlag et al., 2002; Kobayashi et al., 1997). Knockout of ARNT is embryonic lethal due to disrupted placentation and vascular malformation, similar to Hypoxia inducible factor 1 alpha (HIF1α) knockout animals (Ryan et al., 1998). Conditional knockout of ARNT prevents gene regulation by AHR and HIF-1α, confirming a critical role of ARNT in both AHR and HIF-1α signaling (Tomita et al., 2000).

1.2.2.3 Genomic pathway of AHR signalling

Although the transactivation of AHR is well-studied for prototypical AHR target genes such as CYP1A1 and CYP1B1, limited data exist in the genome-wide binding pattern of AHR. The present thesis (Chapter 3 and 4) will aim to address this knowledge gap by providing a comprehensive full genome-wide analysis of AHR binding sites. Such an undertaking will allow us to assess the primary effect (ie. genomic action) of AHR activation and its involvement in biological pathways besides xenobiotic metabolism.

The genomic pathway of AHR signalling describes the mechanism by which AHR ligands bind and activate AHR, leading to receptor-DNA interaction, and ultimately gene regulation. In the absence of ligand, AHR resides in the cytoplasm in a complex with two heat shock protein 90s (hsp90s), X-associated protein 2 (XAP2), and p23 (Kazlauskas et al., 1999; Meyer and Perdew, 1999; Petulis et al., 2003; Petulis and Perdew, 2002). AHR ligands are hydrophobic in nature and diffuse into the cytoplasm to bind AHR. Upon ligand interaction, AHR changes receptor conformation to expose a nuclear localization sequence, which mediates nuclear translocation. AHR can also be activated by a group of atypical
activators independent of direct ligand binding. Omeprazole (OME), a gastric proton pump inhibitor, induces AHRE-driven transcription in an AHR dependent manner (Quattrochi and Tukey, 1993) despite the fact that it does not bind AHR (Backlund and Ingelman-Sundberg, 2004). Mutation analysis indicates that OME-dependent activation of the human AHR is through the phosphorylation of tyrosine 322 (Powis et al., 2011). In agreement with this observation, inhibition of tyrosine kinase effectively attenuates AHR activation by OME (Lemaire et al., 2004).

Once in the nucleus, AHR heterodimerizes with ARNT and binds to specific DNA recognition sites termed the AHREs. AHR binding sites are enriched at promoter regions, but are also distributed into distal enhancer regions as far as 100 kb from transcription start sites (Dere et al., 2011). AHR recruitment to its binding site occurs as early as 15 min after TCDD exposure and peaks at 45 – 60 min in mouse hepatic tissues (Lo et al., 2011). Similarly, 3MC induces maximum AHR recruitment after 30 min of treatment (earliest timepoint examined) and induces oscillatory recruitment of AHR every 1.5 to 2 h in human breast cancer cells in a gene-dependent manner (Pansoy et al., 2010). AHR binding sites typically contain the sequence 5′-GCGTG-3′, where AHR interacts with the 5′GC3′ portion and ARNT, the 5′GTG3′ portion of the core sequence motif (Swanson et al., 1995). In vitro interaction studies of the AHR binding sites around CYP1A1 indicate a strong and stringent requirement of the extended AHRE 5′TnGCGTG3′ (Denison et al., 1988; Swanson et al., 1995). Prior to this thesis, promoter-focused analysis of AHR binding sites in mouse hepatoma cell line and human breast cancer cell line revealed that although AHREs are enriched in AHR binding sites, not all AHR binding site contained the core AHRE (Ahmed et al., 2009; De Abrew et al., 2010; Dere et al., 2011; Sartor et al., 2009). These findings suggest alternative binding sequence or mechanism that might govern AHR-DNA interaction. Two novel response elements have been described that might mediate AHR-DNA interaction in addition to the well-characterized AHRE. They are AHRE-II (5′CATG{N}_{6}CWTG3′) (Boutros et al., 2004; Sogawa et al., 2004) and the non-consensus xenobiotic response element (NC-AHRE; 5′GGGA3′) (Huang and Elferink, 2012). TCDD treatment differentially regulates 13 out of 36 genes containing the AHRE-II (Boutros et al., 2004). The mechanism by which AHRE-II mediates gene regulation is thought to be indirect and might involve an unknown bridging protein (Sogawa et al., 2004). Lastly, Huang et al.
demonstrated AHR interaction with the non consensus-AHRE in the absence of ARNT (Huang and Elferink, 2012), which has not been independently confirmed.

**Figure 6 Canonical pathway of AHR signalling.**

AHR ligands diffuse into the cytoplasm to activate AHR. AHR dissociates from chaperone proteins, exposes its NLS, and translocates into the nucleus. AHR dimerizes with ARNT to bind AHRE in the genome. Negative feedback through AHRR and 26s proteosome-mediated degradation of AHR terminate the signalling pathway.

The AHR signalling pathway is negatively regulated by AHR Repressor (AHRR). AHRR is a poorly understood member of the bHLH-PAS family, whose transient overexpression can attenuate AHR-dependent, AHRE-driven transcription (Mimura et al., 1999). AHR activation induces AHRR expression, thereby initiating a negative feedback mechanism to control AHR activity (Mimura et al., 1999). AHRR appears to repress AHR in a tissue specific context, suggesting additional tissue-dependent players in AHRR-mediated inhibition of AHR activity (Korkalainen et al., 2004). Given the sequence similarity between AHR and AHRR, AHRR might inhibit AHR activity by 1) competing with AHR for ARNT
heterodimerization and 2) AHRR-ARNT heterodimers competing with AHR-ARNT heterodimers for AHRE interaction (Hahn et al., 2009; Mimura et al., 1999). However, over-expression of ARNT does not reverse AHRR-mediated repression, challenging the proposed mechanism through ARNT competition (Evans et al., 2008). Furthermore, an AHRR mutant that lacks the ability to interact with the AHRE, retains its ability to repress AHR (Evans et al., 2008). The precise mechanism of repression by AHRR has yet to be elucidated.

1.2.3 Biological role of AHR

AHR primarily acts as a regulator of xenobiotic metabolism (Bock and Kohle, 2006). It is also the established mediator of TCDD related toxicities. However, its roles in normal physiology and TCDD-related toxicity remain unclear. Recent studies have suggested an important role of AHR in a diverse range of physiological processes including normal vasculature development, organogenesis, fetal development, maintenance of the immune system, and regulator of cell cycle, apoptosis, and differentiation (Bock and Kohle, 2006). TCDD exposure in TCDD-sensitive animals leads to hepatomegaly, thymic involution, cleft palate formation, chloracne, and wasting syndrome (Poland and Knutson, 1982), whereas AHR-null animals are resistant to TCDD-dependent toxicities (Bunger et al., 2008; Bunger et al., 2003). The development of AHR-null or mutant animals reveals several hallmark phenotypes of AHR loss-of-function including patent ductus venosus, reduced liver size, enlarged spleen, and insensitivity to TCDD (Bunger et al., 2008; Bunger et al., 2003; Schmidt et al., 1996).

AHR was first characterized as an inducer of drug-metabolizing enzymes in response to benzo[α]pyrene (B[a]P) and 3-methylcholanthrene (3MC) exposure, leading to substantial up-regulation of cytochrome P450 1A1 (CYP1A1; at the time known as BP hydroxylase or aryl hydrocarbon hydroxylase) (Nebert and Bausserman, 1970b). Since then, a list of AHR regulated genes known as “the AHR gene battery” that facilitates the detoxification and conjugation of potentially toxic xenocheicals has been reported. The AHR gene battery includes Phase I enzymes such as CYP1A1, CYP1A2, and CYP1B1, Phase II enzymes such as NADPH quinone oxidoreductase 1 (NQO1), UDP-glucuronosyltransferase 1A1 (UGT1A1), glutathione s transferase M3 (GSTM3), and drug transporters such as multi-drug resistant protein 4 (MRP4) (Boverhof et al., 2005). This concerted link among Phase I, Phase
II, and drug transporter regulation ensures the conjugation and elimination of harmful reactive intermediates formed from Phase I biotransformation (Kohle and Bock, 2006). This critical interplay between AHR-regulated Phase I and NRF2-regulated Phase II gene expression will be highlighted in Chapter 5.

Two other physiological aspects of AHR signalling – breast cancer and immune disease – have been under intense investigation. AHR activation inhibits E2-dependent proliferation in ER+ MCF-7 breast cancer cells (Chen et al., 1998), and multiple mechanisms have been proposed to mediate its anti-proliferative activity. AHR exerts an inhibitory effect on ERα and has been investigated for its potential as a therapeutic target for hormone dependent breast cancer (Ahmed et al., 2009; Matthews and Gustafsson, 2006; Zhang et al., 2012; Zhang et al., 2009). Secondly, AHR slows cell cycle progression through induction of p21cip1 and p27kip1 to alter cyclin-dependent kinase activity and the phosphorylation of retinoblastoma protein (Rb) (Barnes-Ellerbe et al., 2004; Kolluri et al., 1999). Ligand activated AHR forms a complex with Rb, E2F1, and Dimerization Partner 1 (DP-1) to repress E2F1-regulated genes involved in S phase progression (Marlowe et al., 2004; Puga et al., 2000). Lastly, AHR can inhibit cell proliferation, metastasis, and cell invasion through the regulation of microRNA-335 in hormone independent breast cancer cells (Zhang et al., 2012; Zhang et al., 2009).

Similarly, recent molecular studies have shed light on the mechanism by which AHR controls cell differentiation in the immune system. AHR activation suppresses B-cell differentiation likely through up-regulation of BTB and CNC homology 2 (BACH2) (De Abrew et al., 2010; De Abrew et al., 2011), which is also an NRF2-related transcription repressor. AHR activation by TCDD can influence regulatory T cells (TReg) and T helper 17 cells (Esser et al., 2009). For example, AHR activation by FICZ can promote the expansion of Th17 cell population whereas AHR activation by TCDD can promote the expansion of Treg cell population (Quintana et al., 2008; Veldhoen et al., 2008). However, the precise role of AHR in immunoresponse is beyond the scope of this thesis.
1.3 Nuclear factor erythroid-2 like 2 (NFE2L2; NRF2) and Kelch-like ECH-associating protein 1 (KEAP1)

Nuclear factor erythroid-2 like 2 (NFE2L2; NRF2) belongs to the cap ‘n’ collar (CNC) family of basic leucine zipper (bZIP) transcription factors whereas Kelch-like ECH-associating protein 1 (KEAP1) is a zinc-thiol protein that interacts with NRF2. NRF2 and KEAP1 sense cellular oxidative stress, and in response, regulate the expression of many Phase II detoxification enzymes. The existence of an electrophile/reactive oxygen species (ROS) sensor was first proposed by Dr. Paul Talalay in 1993 when his group investigated the ability of various groups of chemical classes to activate a reporter gene regulated by the antioxidant response element (ARE) (Prestera et al., 1993). NRF2 was initially discovered and cloned in 1994 as a transcription factor that regulates the expression of the beta-globin gene cluster (Moi et al., 1994). However, its role as a master regulator of detoxification enzymes was not apparent until 1996 when Dr. AK Jaiswal’s group demonstrated potent NRF2-mediated regulation of the NQO1 gene containing the human ARE (Venugopal and Jaiswal, 1996). Finally, in 1999, KEAP1, the electrophile sensor and constitutive negative regulator of NRF2 was identified using the yeast two-hybridization assays (Itoh et al., 1999).

1.3.1 Functional domains of NRF2 and KEAP1

The full length NRF2 is located on chromosome 2q31 (chr2: 178,092,323 – 178,257,425) and comprises five exons that together code for a 605 amino acids polypeptide with a predicted molecular weight of 68 kDa. NRF2 can be divided into six conserved domains termed NRF2-ECH homology (Neh) domains. Deletion of the N-terminal Neh2 domains markedly increase the transcriptional activity of NRF2, indicating a negative regulatory role of Neh2. The Neh2 domain mediates interaction with KEAP1 through low affinity DIDLID/DLG (residues 17-32) and high affinity ETGE (residues 79-82) motifs (Itoh et al., 1999). Under unstressed condition, NRF2 forms a complex with two molecules of KEAP1. One molecule of KEAP1 attaches to the ETGE motif of NRF2, while the other KEAP1 molecule latches onto the low affinity DLG motif to lock the NRF2’s lysine-rich helix in place for E2-ligase-catalyzed ubiquitination (Tong et al., 2006). Upon exposure to oxidative stress, the NRF2 DLG motif dissociates from KEAP1 (Tong et al., 2006; Tong et al., 2007), resulting in decreased NRF2 ubiquitination and increased NRF2 stability. NRF2-DNA
interaction and dimerization with sMaf proteins are mediated through the Neh1 domain, which contains the basic DNA binding domain and leucine zipper dimerization domain. A conserved and potentially redox sensitive cysteine residue (C506) in the DNA binding domain is critical for DNA interaction and ARE-mediated gene expression (Bloom et al., 2002). Neh4 and 5 contain the transactivation domain, which is important in the interaction with co-regulators such as CREB binding protein (CBP) and Brahma related gene 1 (BRG1) for gene transcription (Katoh et al., 2001; Zhang et al., 2006). Finally, Neh6 contains a redox-insensitive degradation domain (residues 329-339 and 363-379), which mediates the turnover of active proteins in the nucleus. A dominant negative NRF2 lacking the transactivation domain is sufficient to negatively modulate the activity of NRF2 by competing with NRF2 for sMaf dimerization and ARE-interaction (Alam et al., 1999).

Figure 7 Schematic mapping of functional domains in NRF2.

NRF2 can be divided into six Neh domains. The function associated with each Neh domain is indicated by a line. CNC, Cap N Collar; Neh, NRF2 ECH homology. This figure was modified from (Boutten et al., 2011).

NRF2 contains multiple nuclear export and nuclear localization signals, which regulate nuclear-cytoplasmic shuttling of NRF2. The centrally located Neh5 contains a redox sensitive nuclear export sequence (NES; residues 175 – 186) that induces nuclear
translocation under oxidative stress (Li et al., 2006b). Nuclear export of NRF2 is also facilitated by a redox insensitive NES in the basic leucine zipper motif in the Neh1 domain (Li et al., 2005). Nuclear export of active NRF2 might also be triggered by tyrosine 568 phosphorylation by Src subfamily kinase (Niture et al., 2011). Nuclear translocation is mediated by a bipartite NLS (NLS2) located in the DNA binding domain (residues 494 – 511) (Jain et al., 2005) and two monopartite NLS (NLS1, residues 42-53 and NLS3, residues 587-593) located in the N-terminal Neh2 and the C-terminal Neh3 (Theodore et al., 2008).

**Figure 8 Hinge and latch mechanism of interaction between NRF2 and KEAP1.**

NRF2 interacts with KEAP1 dimer through a low affinity DLG motif and a high affinity ETGE motif. KEAP1 acts as an E2-ligase adapter to facilitate ubiquitination of NRF2. Covalent modification of KEAP1 by electrophiles prevents KEAP1 interaction with DLG motif, disrupting ubiquitination of NRF2.

KEAP1, on the other hand, is a Kelch like protein with two protein interaction domains – BTB (bric-a-brac, tramtrack, broad complex) and DGR-CTR (double glycine repeat C-terminal region) (Itoh et al., 2010). KEAP1 homodimerization is mediated through the BTB domain, while KEAP1 interaction with Neh2, through the DGR-CTR domain (Itoh et al., 1999). Covalent modification of specific reactive cysteines in the intervening region
(IVR; between BTB and DGR-CTR) results in KEAP1-NRF2 dissociation, reduced NRF2 ubiquitination, and NRF2 protein stabilization (Zhang and Hannink, 2003).

1.3.2 NRF2-KEAP1 signal transduction

1.3.2.1 NRF2 activators

NRF2-KEAP1 signalling pathway can be activated by various electrophilic or oxidative compounds with diverse chemical properties (Prestera et al., 1993). However, all NRF2 activators have the ability to react with sulfhydryl groups (Prestera et al., 1993) located in the IVR of KEAP1. NRF2 activators used in a laboratory setting include tert-butylhydroquinone (tBHQ), sulforaphane (SFN), and hydrogen peroxide (H₂O₂); however, there are many dietary, medicinal, and endogenous chemicals that might also be potent activators of NRF2. Sulforaphane, for example, is a dietary isothiocyanate, abundant in the family Brassicaceae (also known as Cruciferae; or commonly referred to as cruciferous vegetables). Cruciferous vegetables contain glucosinolates, which are compounds with a beta-thioglucose moiety, a sulfonated oxime moiety, and a variable amino acid side-chain (Grubb and Abel, 2006). Cleavage of the glucose moiety produces isothiocyanate, an active metabolite that serves as a defense mechanism against insects (Grubb and Abel, 2006). The electron-poor carbon in the isothiocyanate group of sulforaphane can directly form a covalent bond with the sulfhydryl groups on KEAP1 (Hong et al., 2005), rendering the protein inactive. Of the 27 cysteines on KEAP1, electrophiles preferentially bind to C257, C273, C288, and C297 (Dinkova-Kostova et al., 2002). C273 and C288 are required for KEAP1-dependent ubiquitination of NRF2 (Zhang and Hannink, 2003). A third cysteine C151, upon electrophilic modification, allows for NRF2 dissociation (Zhang and Hannink, 2003). Reactive oxygen species (ROS) (Fourquet et al., 2010) and other common electrophilic compounds can activate NRF2, including tBHQ (Zhang and Hannink, 2003), oleanolic acid (Reisman et al., 2009), oltipraz (Ramos-Gomez et al., 2001), and nitrated fatty acid derivatives (Kansanen et al., 2009).
Post-translational modification through protein phosphorylation has also been reported to modulate NRF2 transactivation activity. A comprehensive characterization of kinase-dependent NRF2 activators is currently lacking in the literature. Multiple kinase pathways have been implicated in NRF2 phosphorylation including ERK MAPK kinase-dependent, protein kinase C-dependent, PERK-dependent, and PI3K-dependent pathways (Cullinan et al., 2003; Huang et al., 2002a; Lee et al., 2001; Yu et al., 1999). For example, protein kinase C specifically phosphorylates the serine 40 in the Neh2 domain of NRF2 to promote dissociation from KEAP1 (Huang et al., 2002a). Consistent with this finding, phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, induces ARE-dependent transcription (Huang et al., 2000). The precise mechanism of NRF2 post-translational activation remains elusive and might complement or enhance the transcriptional activity of NRF2. For example, SFN and tBHQ, both KEAP1 inhibitors, can stimulate ERK1/2 MAPK activity, resulting in enhanced ARE-driven transcription (Yu et al., 1999).

1.3.2.2 Genomic pathway of NRF2 signalling

KEAP1 acts as an E3-ligase substrate adapter for NRF2 and constitutively induces NRF2 degradation (Cullinan et al., 2004), resulting in a relatively quick turnover of NRF2 protein with a half-life as short as 15 minutes (Nguyen et al., 2003). Dissociation from KEAP1
initiates NRF2 nuclear translocation and dimerization with small Maf (sMaf) proteins to drive ARE-dependent transcription. sMafs belong to the Maf family of transcription factors. sMaf protein contains a bZip domain that heterodimerizes with the bZip domain of NRF2. NRF2 and sMaf heterodimerization serves two purposes (Li et al., 2008). First, NRF2-sMaf interaction masks the NES domain in the bZip domain of NRF2, disrupting interaction with exportin 1 (CRM-1) and nuclear export (Li et al., 2008). Second, the NRF2-sMaf complex together recognizes a response element termed the antioxidant response element (ARE) (Li et al., 2008). The ARE sequence is deduced using NRF2 target genes from several species including mouse and rat Glutathione S transferase Ya and the rat and human NQO1. It is a relatively flexible motif with the DNA sequence 5′RTGAYnnnGC3′ (Wasserman and Fahl, 1997). Recent genome-wide analyses of NRF2 binding sites in mouse and human revealed similar findings, reporting a DNA sequence of 5′TGACTCAGCA3′ (Chorley et al., 2012; Malhotra et al., 2010). The distribution of NRF2 binding sites is heavily enriched within 5 kb of TSS, although many high confidence binding sites are also located >100 kb from TSS (Chorley et al., 2012). Interestingly, a large number of genes with NRF2 binding sites resulted in no gene expression changes, suggesting other epigenetic regulatory mechanisms governing NRF2 transcriptional activity (Chorley et al., 2012). As NRF2 target genes remove ROS and electrophiles from the cells to re-establish redox homeostasis, NRF2-KEAP1 interaction is restored, effectively terminating the NRF2-KEAP1 signalling pathway.
Figure 10 NRF2-KEAP1 signalling pathway.

NRF2 resides in the cytoplasm in complex with a KEAP1 dimer. Exposure to electrophiles or ROS modified reactive cysteines on KEAP1, resulting the dissociation between NRF2 and KEAP1. NRF2 translocates in the nucleus, heterodimerizes with small Maf transcription factors, and interacts with ARE to regulate Phase II detoxification enzymes. Negative feedback is mediated by SQSTM1, which induces KEAP1 degradation and BACH1, resulting in the formation a non-transcriptionally active complex with NRF2.

Transcriptionally active NRF2 can initiate a positive feedback pathway to enhance its own activity. NRF2 directly regulates the expression p62/SQSTM1 (sequestosome 1) (Chorley et al., 2012; Jain et al., 2010). SQSTM1 contains an ETGE like motif called KEAP1 interacting region (KIR), which binds to the DGR-CTR domain of KEAP1 to disrupt NRF2-KEAP1 interaction (Jain et al., 2010). This positive feedback mechanism allows for sustained activation of the NRF2-KEAP1 signalling pathways upon electrophilic or oxidative challenge.
Similarly, there are negative regulatory controls to balance NRF2 activation. The majority of the negative regulatory controls are mediated through competition for the ARE. For example, sMaf homodimers can compete with NRF2-sMaf complex for ARE interaction. Since sMaf homodimers lack a potent transactivation domain, they form a repressor complex at AREs (Igarashi et al., 1994). Similarly, BACH1, Fos, and Fra1 can all negatively modulate ARE-dependent transcription by dimerizing with sMaf proteins to compete with NRF2 for ARE interaction (Dhakshinamoorthy et al., 2005; Venugopal and Jaiswal, 1996). Despite the fact that KEAP1 has been long recognized as a cytoplasmic, actin-interacting protein (Kang et al., 2004), recent evidence suggests that KEAP1 might shuttle between the nucleus and cytoplasm to repress NRF2 signalling (Nguyen et al., 2005). Nuclear NRF2 are targeted for ubiquitination by the transient nuclear localization of KEAP1 mediated by CRM1/exportin nuclear transport system (Karapetian et al., 2005; Nguyen et al., 2005). Inhibition of CRM1 by lectomycin B leads to substantial KEAP1 nuclear accumulation (Nguyen et al., 2005). The precise mechanism by which KEAP1 enters the nucleus remains unclear since it does not possess a nuclear localization signal (Nguyen et al., 2005).

1.3.3 Physiological role of NRF2 and KEAP1

The primary role of NRF2 is to protect the cell from oxidative insults. Nrf2-null mice were initially developed to probe the effect of NRF2 on erythropoiesis (Chan et al., 1996). Although NRF2 is widely expressed in most tissues and at all stages of embryonic development, Nrf2-null mice are fertile and viable, lacking any discernable phenotypes (Chan et al., 1996). The induction of Nqo1, Gst, and epoxide hydrolase 1 (EH-1) were completely abolished in Nrf2-null mice treated with the antioxidant butylated hydroxyanisole (BHA) (Itoh et al., 1997). Furthermore, Nrf2-null mice are highly susceptible to chemical induced carcinogenesis. Exposure to oltipraz, a NRF2 activator, prevented BaP and N-nitrosobutyl (4-hydroxybutyl)amine (BBN)-induced tumorigenesis in wild type mice, but not in Nrf2-null mice (Iida et al., 2004; Ramos-Gomez et al., 2001). In addition, Nrf2-null mice are susceptible to hyperoxia-induced lung injury (Cho et al., 2002), acetaminophen-induced liver injury (Enomoto et al., 2001; Goldring et al., 2004; Reisman et al., 2009), and cigarette smoke-induced emphysema (Rangasamy et al., 2004).
Keap1 knockout, on the other hand, is post-natally lethal due to hyperkeratosis in the gastric intestinal tract, leading to malnutrition, ulceration of the stomach, and death within seven to ten days after birth (Okawa et al., 2006; Wakabayashi et al., 2003). Cross-breeding with Nrf2-null mice rescues Keap1 deficient mice from hyperkeratosis and post-natal death, indicating a developmental or proliferative role of NRF2 (Wakabayashi et al., 2003). Conditional knockdown of Keap1 in hepatocytes confirms Keap1’s role as a cytoplasmic inhibitor of Nrf2. In the absence of Keap1, Nrf2 localizes in the nucleus, leading to the constitutive up-regulation of Sulfotransferase 2A1/2 (Sult2A1/2), Nqo1, Cyp2b10, Cyp3a11, multidrug resistant protein (Mrp), and glutamate-cysteine ligase (Gcl) (Cheng et al., 2011; Okawa et al., 2006). Organic anion-transporting polypeptide 1A1 (Oatp1a1) expression is attenuated, whereas Oatp1b2 is significantly up-regulated by KEAP1 deletion (Cheng et al., 2011), suggesting a prominent role of KEAP1 and NRF2 in maintaining hepatobiliary transport as well.

NRF2 has been described as a double-edged sword, referring to its protective role as a general antioxidant protein and its pro-survival effect on cancer cells (Hayes and McMahon, 2006; Lau et al., 2008). Many cancer cells possess elevated NRF2 expression and activity (Lau et al., 2008). For example, NRF2 and GST\(\pi\) are significantly up-regulated in hepatoma cells but not in normal hepatocytes (Ikeda et al., 2004). Furthermore, somatic KEAP1 mutations resulting in the lack of NRF2 repression occur at relatively high frequencies in patients with lung adenocarcinoma (Ohta et al., 2008) or breast cancer (Nioi and Nguyen, 2007; Sjoblom et al., 2006). Elevated NRF2 levels can enhance the detoxification and elimination of chemotherapeutic compounds, ultimately compromising the effectiveness of neoplastic agents such as cisplatin, doxorubicin, and etoposide (Lau et al., 2008; Wang et al., 2008). Moreover, NRF2 activation has recently been associated with an increase in cell proliferation and tumorigenicity in non-small cell lung carcinoma (Homma et al., 2009; Singh et al., 2008; Yamadori et al., 2012). NRF2 knockdown sensitizes A549 lung carcinoma cells to cisplatin and induces G\(_1\) cell cycle arrest through a reduction in retinoblastoma phosphorylation (Homma et al., 2009). Interestingly, one of the NRF2 target genes examined in this thesis, heme oxygenase I (HMOX1), might also play a protumorigenic role in cancer cells (Jozkowicz et al., 2007). In summary, the precise
mechanism of NRF2-mediated cell proliferation is incomplete but might involve glutathione-related enzymes, drug transporters, and cell cycle regulators.

1.4 Epigenetic regulation

1.4.1 Epigenetic markers and co-regulatory proteins for ERα

ERα recruitment and its subsequent effects are orchestrated in a concerted manner by multiple co-operative proteins and epigenetic modification (Metivier et al., 2003). Epigenetics describes heritable changes in gene expression independent of DNA sequence. Epigenetic modification of histone protein or DNA can alter local chromatin structure and transcription factor binding and hence modulate gene expression. Functional ERα binding sites are rich in Histone H3 Lysine 4 monomethylation and dimethylation (H3K4Me1 and H3K4Me2 respectively) which determine the recruitment of pioneer factor such as forkhead protein A1 (FOXA1) (Hurtado et al., 2011; Lupien et al., 2008). FOXA1 maintains euchromatic structure of nucleosome-rich chromatin, thereby increasing the accessibility of ERα to DNA (Hurtado et al., 2011). Conversely, CCCTC-binding factor (CTCF) destabilizes FOXA1-DNA and ERα-DNA interaction by decreasing H3K4 methylation, negatively modulating estrogen signalling (Hurtado et al., 2011). In addition to H3K4 methylation, Histone H3 arginine methylation might also influence ERα binding to DNA (Lupien et al., 2009). Coactivator associated arginine methyltransferase 1 (CARM1) methylates arginine residues on Histone H3 and transcriptional coactivators. H3R17Me2 (an indirect measurement of E2-induced CARM1 activity) is associated with increased recruitment of histone acetyltransferases (HATs) such as p300 and nuclear coactivator 1 (NCoA1) and corresponding increase in Histone H3 Lysine 18 acetylation (H3K18Ac), H3K27Ac, and H4K12Ac (Lupien et al., 2009).

ERα binding sites not associated with the epigenetic changes mentioned previously show minimum gene regulation changes (Lupien et al., 2009). These epigenetic changes in the form of histone modification are mediated by a battery of co-regulators. Histone acetylation is mediated by histone acetyltransferases (HAT) or coactivators with HAT
activity and has been proposed to enhance transcription by relaxing local chromatin structure. Proteins with HAT activity include E1A binding protein p300 (p300), cAMP response element binding protein (CBP), HAT1, Tat Interacting protein 60, and the nuclear coactivator family (NcoA) (Zwart et al., 2011). These enzymes mediate the transfer of acetyl moieties to specific lysine residues on the N-terminal of histone proteins. Acetylation of the histone tails neutralizes the positive charge on the lysine residues, thereby decreasing the histone-DNA interaction and relaxing the chromatin.

NCoAs belong to the bHLH family of chromatin modifying proteins and contain three well-defined LXXLL motifs that interact with H12 in the AF2 domain of nuclear receptors (Xu and Li, 2003). Members of the NCoA family have overlapping and redundant functions in co-activating ligand-activated receptor, although individual knockout of each NCoA member displayed differential phenotypes (Xu and Li, 2003). NCoA3 (also known as amplified in breast cancer 1, AIB1; steroid receptor coactivator 3, SRC3; p300/CBP cointegrator-associated protein, pCIP; receptor associated coactivator 3, RAC3; and activator of retinoic acid receptor, ACTR) is the best studied coactivator in estrogen signalling and belongs to the family of transcription coactivator called nuclear coactivators. Genome-wide analysis of NCoA3 revealed a modest overlap (28% and 29%) with ERα and FOXA1 binding sites (Lanz et al., 2010). Proteomic analysis indicated that NCoA3 is the most enriched protein associated with E2-activated ERα in co-immunoprecipitation-mass spectrophotometry (co-IP/MS) (Lanz et al., 2010). Along with ERα, NCoA3 interacts with nucleosome remodeling/histone deacetylase (NURD/HDAC) repressor complex. Interaction between NCoA3 (a transcriptional coactivator) and NURD corepressor complex is a surprise finding. Co-repressors have been demonstrated to enhance transcription in a gene-specific context (Huang et al., 2002b; Perissi et al., 2004; Peterson et al., 2007). This may be mediated by resetting repetitive transcriptional events, although further support is needed.

Whereas coactivators acetylate histones to relax the chromatin, corepressors might condense the chromatin through various epigenetic mechanisms including deacetylation. Gene transrepression might be mediated by the recruitment of histone deacetylases (HDACs), which deacetylate lysine residues on histone proteins to condense chromatin structure. Recruitment of HDACs to repressed genes is facilitated by co-repressors such as
nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT). SMRT and NCoR interact with the AF2 and the DNA binding domain of ERα to repress gene expression (Hu and Lazar, 1999; Varlakhanova et al., 2010), likely by inducing the recruitment of other repressor such as HDAC3 (Privalsky, 2004). Alternatively, repressors such as repressor of estrogen receptor activity (REA) might repress gene expression by competing with other coactivators for interaction with the AF-1 domain on ERα (Delage-Mourroux et al., 2000).

One recent study has systematically screened the requirement of 10 known co-regulators for the expression of six E2 target genes (Won Jeong et al., 2012). Individual RNA interference knockdown of FOXA1, Brahma Related Gene 1 (BRG1), BRG1 associated factor 53 (BAF53), BAF57, Myeloid lymphoid leukemia (MLL1), SET domain containing 1A (SET1A), Tat-interacting protein 60 (TIP60), Flightless-I (FLII), G9a, and nuclear coactivator 3 (NCoA3) significantly affected the ERα-mediated transcription of two estrogen target genes, Gene Regulated in Breast Cancer 1 (GREB1) and Trefoil Factor 1 (TFF1). However, other estrogen target genes such as progesterone receptor (PR) displayed different co-regulator requirement, where FLII, TIP60, BRG1, BAG53, and BAG57 knockdown had no effect on the expression of PR. These findings suggest that the regulation of different estrogen target genes might require different sets of co-regulatory proteins.

1.4.2 Epigenetic markers and co-regulatory proteins for AHR

Like members of the nuclear receptor family, AHR-mediated gene regulation is also facilitated by many co-operative proteins and epigenetic changes. However, AHR transactivation might be associated with its own specific pattern of histone modification. For example, the activation of CYP1A1 was associated with increased Histone H3 serine 10 phosphorylation (H3S10PO4), H3K4Me3, H3K9Ac, H3K14Ac, and H4K16Ac (Schnekenburger et al., 2007). An increase in H3K4Me3 was also associated with a concomitant decrease in H3K4Me2, which might represent a histone methylation event. On the other hand, no changes in the methylation status of H3K9 and H3K27 were observed upon AHR activation (Schnekenburger et al., 2007). Interestingly, significant histone deacetylase 1 (HDAC1) and DNA methyl transferase (DNMT) recruitment was detected
when AHR was not active, suggesting constitutive deacetylation of the CYP1A1 promoter leading to repression of \textit{CYP1A1}. Transient knockdown of HDAC1 increased H3K14Ac and H4K16Ac but was not sufficient to induce CYP1A1 (Schnekenburger et al., 2007). These data indicate that other molecular events in addition to histone modification might govern AHR transactivation. It remains unclear, however, if other AHR target genes exhibit the same pattern of histone modifications upon AHR activation. Chapter 3 addresses the role of histone modification on AHR-dependent gene expression for AHR binding targets other than \textit{CYP1A1}.

The recruitment of co-regulatory proteins to active AHR complexes has been demonstrated to be different in a ligand-specific, cell-line specific, and gene-specific manner, highlighting the flexibility and the functional redundancy of AHR-related co-regulators (Powis et al., 2011; Zhang et al., 2008). Interestingly, there is substantial overlap in the set of co-regulators involved in ER$\alpha$ and AHR signalling pathways, and squelching of shared co-regulatory proteins has been implicated in AHR-ER$\alpha$ crosstalk (Ricci et al., 1999). Co-regulators involved in AHR signalling include the nuclear coactivator (NCoA) family, p300, CBP, receptor-interacting protein 140 (RIP140), CARM1, protein arginine methyltransferase (PRMT1), BRG-1, mediator subunit 200kDa (Med220), Med130, Myb binding protein 1a (Mybbp1a), neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), and promyelocytic leukemia (PML) nuclear bodies (Hankinson, 2005).

Lastly, the function of co-regulators in AHR signalling might be influenced by the co-recruitment of other transcription factors. For example, in the absence of ER$\alpha$ recruitment, RIP140 acts as a co-activator for the transcription of AHR target genes (Madak-Erdogan and Katzenellenbogen, 2012). On the other hand, when AHR and ER$\alpha$ are co-recruited to the same region, RIP140 acts as a transcriptional repressor (Madak-Erdogan and Katzenellenbogen, 2012). This suggests that other co-existing molecular pathways can also modify the function of AHR-related co-regulatory proteins.

\subsection*{1.4.3 Epigenetic markers and co-regulatory proteins for NRF2}

Similar to ER$\alpha$ and AHR, histone acetylation appears to be an important aspect of NRF2-mediated gene transactivation. Ikeda \textit{et al.} reported that acetylation of Histone H3 and
Histone H4 is associated with NRF2-dependent gene expression of the rat GST-p; however, the specific residues that are acetylated were not investigated (Ikeda et al., 2004). H3K9Ac and H3K18Ac positively correlate with NRF2 recruitment and NRF2-dependent ferritin H mRNA expression levels, whereas NRF2 recruitment might inhibit H3K14 acetylation (Sakamoto et al., 2009). Since both transient CARM1 (Histone H3 methyltransferase) and PRMT1 (Histone H4 methyltransferase) over-expression significantly enhance ARE-dependent luciferase activity, it is likely that histone methylation might also be involved in mediating NRF2 gene transactivation (Lin et al., 2006). A systematic and comprehensive investigation of the different histone modification patterns associated with NRF2 activation has not been reported.

Epigenetic control over NRF2 target genes is also mediated by the concerted actions of HATs such as p300/CBP, NCoAs, and HDACs. However, rather than targeting core histones, these co-regulators might modify NRF2 directly to 1) augment transactivation activity and 2) alter protein stability. For example, Sun et al. argued that p300/CBP acetylates specific lysine residues in the Neh1 domain of NRF2 to enhance the transcription of NQO1, GCL, and GSTA1, but not Heme Oxygenase I (HMOX1) (Sun et al., 2009). Furthermore, ARE-dependent luciferase activity is enhanced when NCoA3 and p300/CBP are over-expressed (Lin et al., 2006). On the other hand, increased NRF2 acetylation is associated with decreased stability. Consistent with this observation, treatment with a potent HDAC inhibitor or transient knockdown of HDAC2 significantly reduces NRF2 nuclear accumulation (Mercado et al., 2011). However, it should be noted that none of the studies investigated the role of p300/CBP or HDACs on histone acetylation in intact chromatin. Chapter 5 addresses the epigenetic role of p300 and Histone H3K9Ac in regulating the transcription of NQO1 and HMOX1.
1.5 Interaction among AHR, ERα, NRF2 leading to deregulation of gene expression in breast cancer

1.5.1 AHR modulation of NRF2 activity

1.5.1.1 The effect of AHR activation on NRF2

A recent study by Yeager et al. reported a subset of genes whose expression levels are regulated in a TCDD-dependent, AHR-dependent, and NRF2-dependent manner (Yeager et al., 2009). The genes are appropriately termed “TCDD-inducible, AHR-NRF2 gene battery”. Hayes et al. described at least two potential mechanisms by which AHR regulates the induction of NRF2 target genes (Hayes et al., 2009). The first mechanism is through AHR-dependent up-regulation of NRF2. AHR directly regulates the expression of NRF2 by binding to the AHREs in the NRF2 promoter (Miao et al., 2005), linking Phase I and Phase II gene regulation. For example, AHR activators such as TCDD, βNF, and PAHs are traditionally referred to as bifunctional inducers for their ability to activate both Phase I and Phase II enzyme activity, whereas NRF2 activators such as BHA, oltipraz, and SFN are monofunctional inducers, only capable of activating Phase II activity (Holtzclaw et al., 2004).

Hayes et al. argued against this mechanism since it is unclear how TCDD can remove NRF2 from KEAP1-mediated repression (Hayes et al., 2009). However, NRF2 nuclear accumulation might be a delicate balance between KEAP1-mediated degradation of and de novo synthesis of NRF2 protein (Eades et al., 2011; Nguyen et al., 2005; Stewart et al., 2003). Increase in de novo synthesis of NRF2 protein by AHR might override or saturate the repression by KEAP1, shifting the homeostasis to NRF2 accumulation. Saw et al. (2011) reported synergistic induction of NRF2 target in cells treated with DIM and SFN. Unfortunately, the mechanism for this synergistic induction was not investigated (Saw et al., 2011). Since DIM is a dual activator for both AHR and ERα, we speculate that AHR and ERα might be involved in the synergistic effect observed by Saw et al. Chapter 5 addresses the role of AHR and ERα in the previously reported synergistic induction of NRF2 target genes in DIM and SFN co-treated cells.
The second proposed mechanism is direct interaction between AHR and NRF2, although limited data on physical AHR-NRF2 interaction exist (Hayes et al., 2009). Physical interaction between AHR and NRF2 is conceivable and is probably best illustrated at the regulatory region of NQO1. The regulatory region of NQO1 contains an AHRE and an ARE within 50 bp apart, close enough for physical interaction (Lin et al., 2011). Interestingly, although AHR is recruited to the AHRE upon ligand activation (Yeager et al., 2009), AHRE might be dispensable for the expression of NQO1. Mutation of the AHRE has no effect, whereas mutation of the ARE completely abrogated B[a]P-induced NQO1 expression (Lin et al., 2011). It remains unclear if AHR can physically interact with NRF2 to modulate other NRF2 targets lacking a functional AHRE (Hayes et al., 2009).

1.5.1.2 The effect of NRF2 activation on AHR

Evidence from multiple sources indicates reciprocal crosstalk between AHR and NRF2 and bidirectional signalling between Phase I and Phase II regulation. NRF2 can influence the transcription of AHR, as multiple functional AREs were identified upstream from the TSS of AHR (Shin et al., 2007). Treatment with \{1-[2-cyano-3,12-dioxoolean-1,9(11)-dien-28-oyl]imidazole\} (CDDO-IM), an potent NRF2 activator, induced the constitutive expression of Ahr, Cyp1a1, and Cyp1b1 mRNA in WT MEFs but not in Nrf2-null MEFs (Shin et al., 2007). Furthermore, NRF2 knockout attenuates 3MC-mediated Cyp1a1 induction (Shin et al., 2007). NRF2 might also induce AHR activity indirectly through the induction of HMOX1. HMOX1 is a NRF2 target gene responsible for the catalytic conversion of heme to carbon monoxide, biliverdin, and Fe^{2+}. Biliverdin and bilirubin are both potential endogenous AHR ligands (Denison and Nagy, 2003; Phelan et al., 1998; Sinal and Bend, 1997). Up-regulation of HMOX1 might result in increased production of biliverdin and bilirubin, activating the AHR signalling pathway.

Interestingly, NRF2 activators, such as SFN and SFN-like isothiocyanates elicit cell-specific effects on AHR signalling. SFN and related ITC might act as AHR antagonists, inhibiting AHR nuclear translocation and AHR transactivation in rat liver slices (Abdull Razis et al., 2012a; Abdull Razis et al., 2012b; Skupinska et al., 2009). SFN and phenethyl isothiocyanate (PEITC) prevent B[a]P interaction with AHR (Abdull Razis et al., 2012a; Abdull Razis et al., 2012b). SFN-dependent inhibition of B[a]P-AHR interaction is not
concentration dependent, suggesting non-competitive antagonism through mechanism-based inhibition. This is consistent with the fact that SFN has an electrophilic carbon in its isothiocyanate moiety, which might covalently modify the binding pocket of AHR. However, the role of NRF2 in the SFN-induced inhibition of AHR activity has yet to be determined. On the other hand, SFN might also act as a weak AHR agonist in mouse and human hepatoma cells, capable of inducing AHR-dependent CYP1A1 expression and activity in a dose- and time-dependent manner (Anwar-Mohamed and El-Kadi, 2009). This discrepancy is difficult to reconcile as the experimental conditions and endpoints measured are all very similar with the exception of species differences.

1.5.2 ERα-NRF2 interaction

Breast cancer risk correlates with the duration and concentration of estrogen exposure. Presently, there are two co-existing mechanisms that might interplay to contribute to breast cancer development. Firstly, estrogen can act as a mitogen to increase cell proliferation. Secondly, estrogen can act as a genotoxin to induce DNA damage in a mechanism that might involve E2-dependent repression of NRF2 activity.

1.5.2.1 Estrogen, DNA damage, and detoxification by NRF2

Estrogen can induce DNA damage through two metabolic pathways involving DNA adduct formation by estrogen metabolites and the generation of ROS (Cavalieri et al., 2000). CYP1A1 and CYP1B1 hydroxylate E2 at the C2 and the C4 position to produce catechol estrogens, 2OHE2 and 4OHE2 (Martucci and Fishman, 1993). 2OHE2 and 4OHE2 catechol estrogens, unless detoxified by catechol-O-methyltransferases (COMT), can be oxidized to the reactive 2,3-semiquinone estrogen and 3,4-semiquinone estrogen (Ball and Knuppen, 1980; Yager and Liehr, 1996). Semiquinone estrogens can be further converted into quinone estrogens, which can deplete cellular GSH level (Cao et al., 1998) or react with DNA to produce stable or depurinating adducts (Stack et al., 1996). 3,4-quinone estrogens covalently bind to guanine at N7 position to form 4OHE2-1(α,β)-N7Gua and adenine at N3 position to form 4-OHE2-1(α,β)-N3Ade in vitro and in vivo through direct injection into mammary tissues (Cavalieri et al., 1997). Depurinating adducts result in the formation of apurinic
DNA, which might generate mutations to initiate tumorigenesis. NRF2 target genes such as NQO1 and NQO2 can reduce quinone estrogens into catechol estrogens (Gaikwad et al., 2007; Gaikwad et al., 2009), attenuating the production of E2-DNA adducts.

![Bioactivation and detoxification pathways for genotoxic estrogens.](image)

**Figure 11** Bioactivation and detoxification pathways for genotoxic estrogens.

CYP1A1 and CYP1B1 hydroxylate E2 at the C2 and the C4 position to produce catechol estrogens, 2OHE2 and 4OHE2 (Martucci and Fishman, 1993). 2OHE2 and 4OHE2 catechol estrogens, unless detoxified by catechol-O-methyltransferases (COMT), can be oxidized to the reactive 2,3-semiquinone estrogen and 3,4-semiquinone estrogen (Ball and Knuppen, 1980; Yager and Liehr, 1996). Semiquinone estrogens can be further converted into quinone estrogens, which can deplete cellular GSH level (Cao et al., 1998) or react with DNA to produce stable or depurinating adducts (Stack et al., 1996). Uninterrupted conversion between semiquinone estrogens and quinone estrogens produces an excessive amount of superoxide anion in a process called futile redox cycling (Nutter et al., 1994). Superoxide anion dismutases into hydrogen peroxide which is converted to a potent oxidant, hydroxyl radical in the presence of Fe2+. Hydroxyl radicals oxidize nucleotides to generate mutations and initiate tumorigenesis.
Uninterrupted conversion between semiquinone estrogens and quinone estrogens produces an excessive amount of superoxide anion in a process called futile redox cycling (Nutter et al., 1994). Superoxide anion is converted into hydrogen peroxide by superoxide dismutase, or to hydroxyl radical in the presence of Fe$^{2+}$. Hydroxyl radicals oxidize nucleotides to generate mutations and initiate tumorigenesis. The most abundant and deleterious form of oxidized nucleotide bases is 8-oxo-7,8-dihydro-2’deoxyguanosine (8-oxo-dG) and as such has been routinely used as a marker for oxidative DNA damage (Shigenaga et al., 1994). 8-oxo-dG preferentially pairs with adenine to initiate guanine to thymine transversion mutation (Cheng et al., 1992). In agreement with this proposed mechanism of ROS-dependent breast cancer development, breast cancer patients are susceptible to elevated oxidative stress with significantly higher 8-oxo-dG in sample tissues (Li et al., 2001). As a protective mechanism, NQO1 and NQO2 can disrupt futile redox cycling by catalyzing the reduction of quinone estrogens into catechol estrogens (Gaikwad et al., 2007; Gaikwad et al., 2009).

1.5.2.2 The effect of estrogen receptor activation on NRF2 activity

As described in the previous section, NRF2 target genes play an important role in the detoxification pathway of genotoxic estrogen metabolites. Interestingly, estrogens can repress NRF2 activity, thereby potentiating its genotoxicity. ERα-mediated repression of NRF2 was first reported by Montana et al. who described tamoxifen-dependent up-regulation and estrogen-dependent down-regulation of two NRF2 target genes, NQO1 and GSTYa (Montano and Katzenellenbogen, 1997). Antiestrogens such as ICI and tamoxifen dose-dependently up-regulate NQO1 expression, which is inhibited by E2 co-treatment (Montano and Katzenellenbogen, 1997). Mutation of the AREs upstream of GSTYa attenuates ERα regulation of GSTYa, suggesting that NRF2 and ERα regulate GSTYa through the same cis-regulatory elements (Montano et al., 1998; Montano and Katzenellenbogen, 1997). Follow up experiments also indicate that the effect of antiestrogens is more potent through ERβ (Montano et al., 1998).

Experiments by Ansell et al. shed light on the potential mechanism by which ERα represses NRF2 (Ansell et al., 2005). Using Gal4-NRF2 fusion protein that lacks the ability
to interact with KEAP1 and ARE, Ansell et al. showed that estrogen-dependent repression of NRF2 activity is not mediated through KEAP1 or competition for ARE interaction between ERα and NRF2 (Ansell et al., 2005). ERα-dependent repression of NRF2 is likely attributed to the physical interaction between ERα and NRF2 upon estrogen stimulation (Ansell et al., 2005). Furthermore, the repression can be mediated by either the AF1 domain (Domain A/B) or the DNA binding domain (Domain C) (Ansell et al., 2005). Yao et al. recently revealed that ERα represses NRF2 by facilitating the recruitment of SIRT1, a class III histone deacetylase, to NRF2 binding sites (Yao et al., 2010). Collectively, these findings imply that ERα represses NRF2 target genes, which has implication in the etiology of breast cancer since many NRF2 target genes such as NQO1, NQO2, GST, and GCLC are involved in the detoxification of genotoxic estrogen metabolites.

1.5.2.3 The effect of NRF2 activation on ERα activity

While ERα imposes a general inhibitory effect on NRF2, the effect of NRF2 on ERα activity has not drawn as much attention. Exposure to SFN, a NRF2 activator, down-regulates ERα mRNA expression and induces ERα protein degradation in a dose- and time-dependent manner, ultimately leading to the induction of apoptosis in MCF-7 ERα positive breast cancer cells (Pledgie-Tracy et al., 2007; Ramirez and Singletary, 2009). Unfortunately, the authors did not investigate the role NRF2 plays in SFN-dependent down-regulation of ERα mRNA and protein level (Pledgie-Tracy et al., 2007; Ramirez and Singletary, 2009). Given that NRF2 directly up-regulates AHR expression (Shin et al., 2007) and that AHR activation degrades ERα protein (Wormke et al., 2003), it is conceivable that SFN-induced ERα protein degradation involves both AHR and NRF2, highlighting a tight regulatory network among ERα, AHR, and NRF2 in breast cancer.

1.6 Study Rationales

Evidence to date suggests extensive interaction among AHR, NRF2, and ERα. AHR activation up-regulates Phase I metabolic enzymes such as CYP1A1 and CYP1B1 to bioactivate E2, whereas NRF2 activation up-regulates detoxifying enzymes such as NQO1,
GSTYa, and GCLC to detoxify and eliminate those genotoxic E2 metabolites. ERα activation can repress NRF2 target genes and thereby can further enhance the formation of genotoxic metabolites to induce mutagenesis. ERα can also mediate the mitogenic effect of E2, ultimately promoting the proliferation of mutated cells in the development of breast cancer. Hence, a comprehensive investigation of the genomic transcriptional targets of ERα, AHR, and NRF2 might identify additional molecular mechanisms involved in breast cancer development. This thesis will address how AHR, ERα, and NRF2 might independently or synergistically modulate Phase I and Phase II enzymes, potentially deregulating the production and elimination of genotoxic estrogen metabolites in the initiation of breast cancer.

To address the role of ERα, AHR, and NRF2 in breast cancer, I have three research aims:

1) Investigate genome-wide ERα binding sites in T-47D breast cancer cells and potential cell line differences between T-47D and the commonly used MCF-7 breast cancer cells.

2) Investigate genome-wide AHR binding sites in vivo and in vitro.

3) Investigate how the genomic activation of ERα and AHR affects the transcriptional activity of NRF2 in the regulation of NQO1 and HMOX1.

1.6.1 Aim 1: Investigate genome-wide ERα binding sites in T-47D breast cancer cells and potential cell line differences between T-47D and the commonly used MCF-7 breast cancer cells

Recent chromatin immunoprecipitation (ChIP) combined with microarrays (ChIP-chip) have identified several ERα-bound genomic regions at a genome-wide level (Carroll et al., 2006; Kwon et al., 2007). Most genome-wide analyses of ERα binding sites have been done using ERα-positive MCF-7 human breast cancer cell line (Carroll et al., 2006; Kininis and Kraus, 2008; Kwon et al., 2007; Lin et al., 2007) with little information about ERα binding profiles in other ERα-positive human breast cancer cell lines. Given potential cell line differences in estrogen signalling (Hurtado et al., 2011; Kininis and Kraus, 2008; Lo et al., 2010), it is
important to compare the genome-wide binding of ERα from multiple breast cancer cell
lines. To determine genome-wide ERα binding sites in T-47D breast cancer cells, we
performed promoter-focused chromatin immunoprecipitation followed by microarray chip
(ChIP-chip) in asynchronously growing T-47D cells in normal fetal bovine serum, which
contains approximately 0.1 nM E2. Comparison between the multiple cell lines in terms of
genomic ERα binding sites and regulation targets should give us a better representation of
ERα-dependent transcription in normal mammary cells. Aim 1 of the thesis is presented in
Chapter 2 Estrogen receptor-dependent regulation of CYP2B6 in human breast cancer cells,

1.6.2 Aim 2: Investigate genomic AHR binding sites in vivo and in vitro

AHR plays an important role as the primary regulator of xenobiotic metabolism and
activation of E2 into genotoxic estrogen metabolites. Therefore, a comprehensive analysis of
the mechanism by which AHR regulates its target genes is needed. Recent ChIP-chip
experiments have identified many AHR-bound genomic regions in vitro with little
information about AHR genomic binding pattern in vivo (Ahmed et al., 2009; De Abrew et
al., 2010; Pansoy et al., 2010; Sartor et al., 2009). Given the well-established differences in
AHR signalling in vivo and in vitro (Dere et al., 2006), it is important to characterize the
genomic AHR binding in vivo and the epigenetic factors that govern its transcriptional
activity. This aim is presented in Chapter 3 Identification of AHR binding targets in mouse

Initial ChIP-chip experiments were conducted in mouse liver because it is the primary target
tissue of AHR and because there are corresponding AHR ChIP-chip datasets in hepatoma
cell lines in the literature for direct comparison. To investigate whether AHR behaves
similarly as the primary regulator of xenobiotic metabolism in breast cancer and to better
understand the role of AHR and ARNT signalling in breast cancer, I performed a follow-up
experiment to investigate genome-wide AHR and ARNT binding in human MCF-7 breast
cancer cells using ChIP-Seq. Genome-wide characterization of the genomic action of AHR
and ARNT is presented in Chapter 4 high-resolution genome-wide mapping of AHR and ARNT binding sites by ChIP-Seq, which is in press in *Toxicological Sciences*.

### 1.6.3 Aim 3: Investigate how ERα and AHR might affect the transcriptional activity of NRF2

ERα and AHR have both been reported to modulate the transcriptional activity of NRF2 with ERα having an inhibitory effect and AHR having a positive effect. However, no studies have investigated how the simultaneous activation of AHR and ERα might affect NRF2 activity. To this end, I measured expression levels of NQO1 and HMOX1 to evaluate NRF2 transcriptional activity in MCF-7 breast cancer cells treated with DIM (a dual AHR and ERα activator) and SFN (NRF2 activator). ChIP assays were conducted to elucidate the transcription factors, co-regulatory proteins, and epigenetic modifications required for enhancing or repressing NRF2 transcriptional activity. This study is presented in Chapter 5 and is submitted for peer-review. This study improved our understanding of the link between Phase I and Phase II gene regulation through interaction among AHR, ERα, and NRF2 and shed light on how AHR and ERα activation might influence NRF2 activity and hence the production of estrogen metabolites in the genotoxic pathway of breast cancer development.
Chapter 2  Estrogen receptor-dependent regulation of CYP2B6 in human breast cancer cells

Raymond Lo, Lyle Burgoon, Laura MacPherson, Shaimaa Ahmed, and Jason Matthews

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All experiments were conducted by Raymond Lo with the exception of the small interference RNA experiments, which were conducted by Laura MacPherson and the chromatin immunoprecipitation-on-microarray chip experiments, which were conducted by Jason Matthews. Data analyses were performed by Raymond Lo, Lyle Burgoon, and Jason Matthews. The study was designed by Jason Matthews. The final manuscript was written by Raymond Lo and Jason Matthews.

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Abstract

In this study, we used chromatin immunoprecipitation and promoter focused microarrays (ChIP-chip) to identify estrogen receptor α (ERα) binding sites in T-47D human breast cancer cells. The isolated regions included known and several new ERα-bound regions, including multiple sites upstream of the CYP2B gene cluster. Since previous studies have reported a correlation between ERα levels and CYP2B6 expression, we characterized the ERα recruitment to the genomic regions in the 5' enhancer region of CYP2B6. Reporter gene assays revealed ERα-dependent regulation of CYP2B6 through a functional upstream estrogen response element (ERE). E2 increased ERα and coactivator recruitment to the CYP2B6 promoter ERE, and increased CYP2B6 mRNA levels in T-47D but not MCF-7 human breast cancer cells. RNAi-mediated knockdown of ERα in the T-47D cells resulted in a significant decrease in CYP2B6 mRNA levels. Overall, this study provides the first demonstration of direct transcriptional activation of the CYP2B6 gene by ERs in a cell line-specific manner.
Introduction

Estrogens act through their nuclear receptors, estrogen receptor α (ERα; NR2A1) and ERβ (NR3A2) to regulate diverse transcriptional responses that include both stimulation and repression of gene expression (Nilsson and Gustafsson, 2002). ERs share the evolutionarily conserved functional domains typical of other nuclear receptor family members (Ruff et al., 2000). These include the amino-terminal activation function-1 (AF1) domain, the centrally located DNA-binding domain (DBD) and the carboxy-terminal ligand-binding domain (LBD), which also contains the ligand-dependent activation function-2 (AF2) region that is important for interaction with coactivators (Nilsson et al., 2001). Ligand binding induces conformational changes in the receptors resulting in homodimerization and subsequent DNA binding. ER homodimers bind to perfect and imperfect palindromic DNA sequences termed estrogen responsive elements (EREs) located in the regulatory region of their target genes. Once bound to DNA, ERs act as nucleation sites for the recruitment of chromatin remodeling complexes and co-regulators (i.e. nuclear receptor coactivator 1-3, p300/Creb binding protein), and the assembly of basal transcriptional machinery (Sanchez et al., 2002). ERs also regulate transcription via half-site EREs and by a tethering mechanism that involves protein:protein interactions with other DNA-bound transcription factors, including activating protein 1 and stimulating protein 1 (Sanchez et al., 2002).

Estrogens are critical for the growth, development and differentiation of the mammary gland. ER expression in breast cancer is associated with improved responsiveness to endocrine targeted therapies, such as treatment with the anti-estrogen tamoxifen to reduce ER activity or treatment with aromatase inhibitors to deplete estrogen levels (Dutta and Pant, 2008). In addition to increasing the expression of genes that regulate cell proliferation and cancer development, ERα has also been reported to regulate the expression of many drug metabolizing enzymes, including cytochrome P450 1A1 (CYP1A1), CYP1B1, and CYP2A6 (Higashi et al., 2007; Matthews et al., 2005; Tsuchiya et al., 2004). Two recent studies have reported higher CYP2B6 expression in ERα-positive compared to ERα-negative breast tumors (Bieche et al., 2004; Tozlu et al., 2006), implicating CYP2B6 as an ERα target gene.
Hepatic CYP2B genes are the most inducible CYP isoforms by phenobarbital (PB)-type inducers. CYP2B6 is expressed in human liver, where it constitutes approximately 6% of total microsomal P450s (Stresser and Kupfer, 1999), but it is also expressed in extrahepatic tissues including intestine, kidney, lung, skin, brain and mammary gland (Ding and Kaminsky, 2003; Janmohamed et al., 2001; Miksys et al., 2003). CYP2B6 metabolizes a wide variety of pharmaceutical agents including cyclophosphamide, buproprion and tamoxifen; environmental contaminants such as aflatoxin B and dibenzanthracene; and nicotine and methylenedioxymethamphetamine (MDMA “ecstasy”) (Hodgson and Rose, 2007; Wang and Tompkins, 2008). CYP2B6 expression is induced through proximal and distal regulatory regions at -1.7 and -8.5 kb via the constitutive androstane receptor (CAR; NR1I3), and hepatic expression of CAR correlates with CYP2B6 expression (Wortham et al., 2007). Upon nuclear translocation, CAR associates with its dimerization partner retinoid X receptor (RXR; NR2B1). The CAR-RXR complex binds the phenobarbital response enhancer module (PBREM) and recruits co-regulator proteins to modulate target gene expression, such as  CYP2B6 (Sueyoshi et al., 1999). Although CAR has long been recognized as a key regulator of  CYP2B, emerging evidence suggests that other nuclear receptors such as pregnane X receptor (PXR; NR1I2) (Goodwin et al., 2001) and glucocorticoid receptor (GR; NR3C1) (Audet-Walsh and Anderson, 2009), and liver enriched transcription factors are also involved in the regulation of this gene (Pascual et al., 2008). Interestingly, many of the potent inducers of CYP2B6 expression are ligands for PXR but not CAR (Faucette et al., 2007). Putative glucocorticoid response elements (GREs) are present in the upstream regulatory regions of mouse  Cyp2b10 and rat  CYP2B1/2, that are responsive to the synthetic glucocorticoid, dexamethasone (DEX) (Honkakoski et al., 1996; Stoltz et al., 1998). DEX treatment has been reported to be required for maximum induction of Cyp2b10 expression by activators of CAR (Honkakoski et al., 1996). It is unclear if this also occurs in humans, since the CYP2B6 promoter region does not contain a GRE. However, activation of GR has been reported to increase the expression of PXR and CAR in human hepatocytes (Pascussi et al., 2000). High doses (μM) of 17β-estradiol (E2) activate mouse but not human CAR; however, direct regulation of  CYP2B6 by ERs has not been determined.
Recent chromatin immunoprecipitation (ChIP) combined with microarrays (ChIP-chip) have identified several ERα-bound genomic regions at a genome-wide level (Carroll et al., 2006; Kwon et al., 2007). Most genome-wide analyses of ERα binding sites have been done using ERα-positive MCF-7 human breast cancer cell line (Carroll et al., 2006; Kininis and Kraus, 2008; Kwon et al., 2007; Lin et al., 2007) with little information about ERα binding profiles in other ERα-positive human breast cancer cell lines. In the present study, we did ChIP-chip to identify ERα-bound genomic regions in T-47D human breast cancer cells. One of the ERα-bound regions identified was located in the 5′-regulatory region of CYP2B6. Reporter gene assay, mRNA expression, and protein expression analysis provide evidence for ER-dependent regulation of CYP2B6.
Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), 17β-estradiol (E2) and ICI 182,780 were purchased from Sigma (St. Louis, MO). Primers for polymerase chain reaction (PCR) were purchased from Integrated DNA Technology (Coralville, Iowa, USA). Cell culture media, fetal bovine serum (FBS) and trypsin were purchased from Wisent (St. Bruno, Canada). All other chemicals and biochemicals were of the highest quality available from commercial vendors.

Plasmids

To generate pGL3p-2B7P, Region_6 from the array (chr19: 46,118,736 to 46,119,920) was amplified from isolated T-47D genomic DNA and cloned into the KpnI and BglII sites of pGL3 promoter vector. Region_6 is 69 kb upstream from CYP2B6, but also maps approximately 10 kb upstream from CYP2B7P. To generate pGL3-2B6, we have amplified the -1.8 kb 5’ flanking region of CYP2B6 promoter (chr19: 46,187,266 to 46,189,032) from T-47D genomic DNA. This sequence includes region_42 (chr19: 46,187,186 to 46,187,511) identified from our ChIP-chip experiments. The -1.8 kb 5’ flanking region was cloned into the KpnI and BglII sites of pGL3 basic vector to yield the plasmid pGL3-2B6. pGL3-2B6 ERE mut was created by site directed mutagenesis using the following primers to destroy the putative ERE: 5’-GCTCCTCCTGTTCAAAGTAAC-3’ and 5’-GTTACTTTGAAAACAGGAGGAGC-3’ in Region_42. For the plasmid pGL3-2B6 ΔERE, a new KpnI site 3’ of the ERE was introduced to pGL3 2B6-ERE using the following primers: 5’-CAGGTCCTGATTCCAGGACCTG-3’ and 5’-CCTTTGCTGGTACCAGGACCTG-3’. KpnI was then used to cut out a 156 bp DNA fragment containing both the PBREM and putative ERE. Subsequent ligation yielded the plasmid pGL3-2B6 ΔERE. pSG5 ERα and pSG5 ERβ were generous gifts from Prof. Jan-Åke Gustafsson (University of Houston, TX, USA). For the plasmid pSG5 ERα DBD, E203A and G204A mutations were introduced to the DNA binding domain of ERα using the following primers: 5’-
GTCTGGTCCCTGCGCCTGCAAGGCCTTC-3’ and 5’-GAAGGCCTTGCAGGCCGCACAGGACCAGAC-3’. All mutations were verified by DNA sequencing.

**Cell culture**

T-47D human breast carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium and F12 medium in a 1:1 mixture ratio (DMEM:F12), supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PEST). HuH-7 human hepatoma cells were cultured in high glucose DMEM supplemented with 10% FBS and 1% PEST. MCF-7 human breast carcinoma cells were cultured in DMEM, supplemented with 10% FBS and 1% PEST. In experiments investigating the effect of E2, the cells were plated in phenol red free media, supplemented with 5% dextran-coated charcoal (DCC) treated FBS for 72 h before transient transfection. All cells were maintained at 37°C in a 5% CO₂ environment and subcultured at 80% confluency.

**Chromatin immunoprecipitation and ChIP-chip**

ChIP assays were performed as described by Matthews et al. (Matthews et al., 2005). Chromatin was sonicated to an average size of 500 bp. T-47D and MCF-7 cells were grown in phenol red free media supplemented with 5% DCC-FBS and 1% PEST for 3 days and treated with either 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780 for 1 h or 4 h. The isolated chromatin was incubated with 0.8 μg of either normal rabbit IgG, anti-ERα (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-NCoA3 (M-397; Santa Cruz). The isolated DNA fragments were quantified using quantitative real-time PCR (qPCR).

For the ChIP-chip experiments, T-47D cells were plated in 10-cm dishes in DMEM:F12 supplemented with 10% FBS and 1% PEST. After 48 h, ChIP assays were performed as described previously (Matthews et al., 2005). Immunoprecipitated DNA from 10 cm/antibody was linearly amplified using Primer A: GTTTCCCAGTCACGTC(N)₉ and
Primer B: GTTTCCCAGTCACGGTC according the Affymetrix random hexamer linear amplification protocol. Linearly amplified DNA (7.5 μg) was fragmented by limited DNAsel digestion and hybridized to Affymetrix human promoter tiling arrays 1.0R (Affymetrix, Santa Clara, CA). Hybridization and washing steps were performed according to the manufacturer’s protocol. Data were normalized and analyzed using CisGenome (Ji et al., 2008). Enriched peaks were determined by comparing the triplicate ERα samples to IgG using TileMap v2 by a moving average approach using default settings (Ji and Wong, 2005). Regions were merged if the gap between them was <300 bp and the number of probes failing to reach the cut-off was <5. Regions were discarded if they were <120 bp or did not contain at least 5 continuous probes above the cut-off.

**Cis-Regulatory Element Search**

To calculate the enrichment of individual cis-regulatory elements, we performed a position weight matrix (PWM) search across the ChIP-enriched sequences using an in-house cis-regulatory element search application. Our application was written in Ruby (v 1.8.6) and used the RinRuby package for connectivity to the R statistical interpreter (v 2.8.1). We first searched all of the 243 enriched sequences at 1% FDR using all of the JASPAR (http://jaspar.cgb.ki.se/) PWMs for human, mouse, rat or undeclared species (i.e., denoted as "-"). Our algorithm defined putative binding sites as those within a ChIP-chip site that exceeded our matrix similarity score cut-off of 0.80. For comparison purposes, the application also counted the average number of times a putative cis-regulatory element occurred within 100 randomly generated sequences based on the same nucleotide frequencies as the ChIP-chip site. Site-based and whole dataset enrichment probability scores were calculated using a Bayesian probabilistic method. For the site-based enrichment probability, we used the cumulative rather than regular probability for ease of biological interpretation. For the whole dataset enrichment, we calculated the total counts from all of the ChIP-chip sites and the total random counts from all of the sites, and performed Poisson-based probability calculations.
Transient transfection and reporter gene assay

HuH-7 cells were plated in 12-well dishes in DMEM medium containing either 10% FBS or 5% DCC-FBS containing phenol red free DMEM. 24 h after plating, the cells were transfected with pSG5 ERα, pSG5 ERβ, and luciferase reporter vectors using Lipofectamine 2000 (Invitrogen Corp., Burlington, Canada). The cells were dosed 24 h post-transfection with either 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780. The following day, cells were lysed and luciferase activity was determined according to the manufacturer’s instructions (Promega, Madison, WI). The firefly luciferase activity was normalized to that of the renilla luciferase and the normalized data was then presented relative to empty vector control (Promega).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

T-47D and MCF-7 cells were dosed for 6 h with either 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780. RNA was isolated using RNeasy Mini Kit (Qiagen, Mississauga, Canada) as described by the manufacturer. Half a microgram of the isolated RNA was reversed transcribed using random hexamer primers and SuperScriptII Reverse Transcriptase (Invitrogen). The cDNA was amplified with the appropriate primers, and quantified using SYBR green (Bio-Rad Laboratories, Mississauga, Canada).

Western blot analysis

Western blots were performed as described previously (Ahmed et al., 2009). Antibodies used were anti-ERα (HC-20; Santa Cruz), anti-β-actin antibody (Sigma) and anti-CYP2B6 antibody (AB1283; Chemicon International, Temecula, CA). Enhanced chemiluminescence (ECL) reagents from GE Healthcare (Mississauga, Canada) and film from Denville Scientific (Saint-Laurent, Canada) were used to detect protein.
**ERα knockdown and small interfering RNA**

ERα (L-003401-00-0020) ON-TARGETplus SMART pool small interfering RNA (siRNA) and DharmaFECT1 transfection reagent were purchased from Dharmacon (Lafayette, CO). ERα knockdown experiments were performed as described previously (Ahmed et al., 2009). Briefly, T-47D cells were seeded 300,000 per well in six-well plates containing 2 ml of medium. After 24 h, 100 nM of siRNA against ERα (L-003401-00-0020) or nontargeting pool (NTP) (D-0011810-10-20) (Dharmacon) were transfected using 4 µl of DharmaFECT. ChIP assays and mRNA isolation were prepared 48 h after transfection.

**Statistical Analysis**

All results are expressed as means ± standard error of the means (SEM). Statistical analysis was calculated using GraphPad Prism 5 statistical software (San Diego, CA). One-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison tests and the Student’s two tailed $t$-tests were used when appropriate. Statistical significance was assessed at $p < 0.05$. 
Results

Estrogen Receptor Binding Sites in T-47D cells

To identify ERα binding sites in T-47D human breast cancer cells, we performed ChIP-chip assays on T-47D cells plated for 48 h in 10% FBS complete serum. ERα was active under these conditions due to residual levels of estrogens in the serum (Murphy et al., 1989). ELISA assays showed that the E2 concentration in our complete medium was approximately 0.1 nM. Chromatin was isolated using either an anti-ERα antibody or normal rabbit IgG. Isolated and linearly amplified DNA was hybridized to Affymetrix Human tiling 1.0R microarrays. We performed three biological replicates and data were normalized and analyzed as described in the Materials and Methods. We identified 243 promoter regions bound by ERα using a false detection rate (FDR) of 1%. The identified regions were assigned a relative rank (region number correlates with statistical significance such that region 1 represents the highest ranked region within the data set) based on a statistical analysis of the data performed in CisGenome (Ji et al., 2008). We then compared our ERα-bound regions in T-47D cells to those identified in a genome-wide analysis in MCF-7 (Carroll et al., 2006). Only 88 regions (36%) overlapped with at least 50% sequence identity between the two data sets (Figure 12). This relatively low level of overlap could be due to differences in experimental conditions, but also might reflect cell-specific differences between the two breast cancer cell lines.

Figure 12 ERα-bound regions in T-47D cells.

Comparison of the ERα-bound regions identified in the current study (T-47D) with those reported in a genome-wide analysis in MCF-7 cells (Carroll et al., 2006). As shown in the venn diagram 88 of the 243 regions identified in our study overlapped with at least 50% sequence identity to those reported previously (Carroll et al., 2006).
To determine which *cis*-regulatory elements were significantly over-represented in the isolated ChIP-chip regions, we performed a position weight matrix (PWM) search across the ChIP-enriched sequences. Our analysis identified 36 transcription factor motifs that were significant at $p < 0.01$. The ESR1 binding motif, or ERE as defined in the JASPAR database, was significantly over-represented in the ERα-bound regions. Overall, approximately 50% of the 243 regions contained an ERE.

### Table 1

Transcription factor motifs that were over-represented in the ERα-bound regions identified in T-47D. Table shows the top ranking transcription factor motifs.

<table>
<thead>
<tr>
<th>Binding motif a</th>
<th>Sum of Real Count</th>
<th>Random Count</th>
<th>Over representation</th>
<th>Poisson p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR1</td>
<td>162</td>
<td>25.23</td>
<td>6.42</td>
<td>0.0</td>
</tr>
<tr>
<td>Myf</td>
<td>427</td>
<td>192.85</td>
<td>2.21</td>
<td>0.0</td>
</tr>
<tr>
<td>NHLH1</td>
<td>305</td>
<td>143.07</td>
<td>2.13</td>
<td>0.0</td>
</tr>
<tr>
<td>IRF2</td>
<td>110</td>
<td>51.69</td>
<td>2.13</td>
<td>5.50E-13</td>
</tr>
<tr>
<td>Hand1-Tcfe2a</td>
<td>2355</td>
<td>1228.77</td>
<td>1.92</td>
<td>0.0</td>
</tr>
<tr>
<td>Foxd3</td>
<td>814</td>
<td>433.79</td>
<td>1.88</td>
<td>0.0</td>
</tr>
<tr>
<td>Roaz</td>
<td>376</td>
<td>203.67</td>
<td>1.85</td>
<td>0.0</td>
</tr>
<tr>
<td>NR2F1</td>
<td>138</td>
<td>76.91</td>
<td>1.79</td>
<td>1.28E-10</td>
</tr>
<tr>
<td>HNF4A</td>
<td>378</td>
<td>212.63</td>
<td>1.78</td>
<td>0.0</td>
</tr>
<tr>
<td>FOXI1</td>
<td>1830</td>
<td>1035.8</td>
<td>1.77</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* Refer to JASPAR (http://jaspar.cgb.ki.se/) for detail descriptions of the transcription factor motifs.
**Confirmation of ChIP-chip results**

To verify the results obtained from the ChIP-chip analysis and determine the E2-dependent recruitment of ERα to the regions identified from the analysis, we performed conventional ChIP on T-47D cells grown for 3 days in steroid-deprived medium before treatment with 10 nM E2 for 1 h. We selected 13 ERα-bound regions to further investigate. The binding regions were chosen to cover a range of enrichment values but also included known ERα-regulated genes. E2-dependent recruitment of ERα was confirmed to all 13 regions examined, although the fold enrichments varied among the regions (Figure 13A). Some of the identified regions included known estrogen target genes such as gene regulated in breast cancer 1 (GREB1), RAS-like estrogen-regulated growth inhibitor (RERG) and estrogen receptor α (ESR1) (Figure 13B). Surprisingly, ERα binding to trefoil factor 1 (TFF1), a gene that is routinely used to study ERα-mediated transcription (Metivier et al., 2003), was not detected under our assay conditions. Several of the identified ERα-bound regions have not been previously reported in ChIP-chip studies using MCF-7 cells (Carroll et al., 2006; Kwon et al., 2007; Lin et al., 2007), including region_6 and region_42 that map to the 5’ regulatory region of CYP2B7P and CYP2B6, respectively (Figure 13B). Since CYP2B6 is involved in the metabolism of several clinically important drugs and it has been reported to correlate with ERα expression (Tozlu et al., 2006), we decided to investigate the possible ER-dependent regulation of CYP2B6 expression.
Figure 13 Confirmation of ERα-bound regions identified in the ChIP-chip study.

(A) Conventional ChIP was performed to confirm 13 ERα-bound regions in T-47D plated in DCC-FBS containing medium and treated with E2 for 1 h. These 13 regions were chosen to represent a range of enriched values from the array. ERα recruitment level significantly (p < 0.05 Student’s t-test) different from DMSO is indicated by an asterisk for each region. (B) Selected ERα-bound regions relative to their closest genes are shown using USCS Genome Browser (http://genome.ucsc.edu/). The black blocks represent ERα-bound regions identified from our ChIP-chip study, whereas the arrows indicate the transcriptional direction of the closest annotated genes. (C) The putative EREs in the 5’ upstream regulatory region of CYP2B6. The diagram shows the genomic coordinates and the sequences of the putative EREs in Region_6 and Region_42 (including the flanking regions and the nearby PBREMs). The perfect palindromic ERE from vitellogenin A2 is also shown for comparison. NCBI Blast 2 sequences indicates >80% homology among the sequences. A colon indicates homology among the sequences, where an underline indicates at least one dissimilarity among the sequences at that position.
**ER expression positively correlates with CYP2B6 levels**

To investigate ER-dependent regulation *CYP2B6*, we cloned a ~1.8 kb fragment upstream from the *CYP2B6* start site, which included region_42. Transcription factor binding site analysis identified a putative ERE located approximately -1.7kb from the *CYP2B6* start site. The putative ERE is an imperfect palindromic sequence (GGTCAnnnTAACT) compared to the perfect ERE (AGGTCAnnnTGACCT) in the upstream regulatory region of vitellogenin A2 (Klein-Hitpass et al., 1986). The putative ERE is approximately 40 bp downstream of the PBREM. Comparative genomic analysis revealed that the ERE present in the regulatory region of *CYP2B6* was not conserved in the 5′ regulatory region of the mouse homolog *Cyp2b10* nor the rat homolog *Cyp2B1*. Region_6 contained three putative EREs with the same sequences as the ERE in region_42 (Figure 13C).

To investigate the transcriptional activation of *CYP2B6* by ERs, luciferase reporter assays were performed (pGL3-2B6) with increasing amounts of ERα or ERβ in ER-negative HuH-7 human hepatoma cells. The reporter activity of pGL3-2B6 (Figure 14 A and C) or pGL3p-2B7P (Figure 14 B and D) increased with increasing amounts of ERα or ERβ. To determine the E2-dependent regulation of *CYP2B6*, HuH-7 cells were plated in steroid-deprived medium and pGL3-2B6 and pGL3p-2B7P were transiently transfected with fixed amounts of ERα or ERβ. In HuH-7 cells transfected with either ERα or ERβ, treatment with 10 nM E2 caused a significant increase in luciferase activity for both pGL3-2B6 (Figure 14 E and G) and pGL3p-2B7P (Figure 14 F and H), which was inhibited by co-treatment with the ER antagonist, ICI 182,780.
Figure 14 ER-dependent transcriptional activation of pGL3-2B6 and pGL3p-2B7P in HuH-7 cells.

HuH-7 cells were transfected with (A) 200 ng of pGL3-2B6 or (B) pGL3p-2B7P and increasing amounts of the plasmid pSG5 ERα for 24 h prior to luminescence detection. pGL3-2B6 activity is regulated by the natural promoter of CYP2B6, whereas pGL3p-2B7P, by the regulatory region located 69 kb upstream of CYP2B6, which also maps to the 5' regulatory region of CYP2B7P. Results shown are means ± S.E.M. for three independent experiments. Similar results were obtained when increasing amounts of the plasmid pSG5 ERβ were transfected with the two reporter gene plasmids (C and D). In order to evaluate the estrogen responsiveness of CYP2B6, HuH-7 cells were plated in DCC-FBS containing medium for 24 h. Cells were co-transfected with (E) 200 ng of pGL3-2B6 and 50 ng of pSG5 ERα or (F) 200 ng of pGL3p-2B7P and 5 ng of pSG5 ERα for 24 h. The cells were treated with either 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780 or 10 nM E2 + 100 nM ICI 182,780 for an additional 24 h prior to luminescence detection. Similar results were obtained when (G) 200 ng of pGL3-2B6 and 100 ng of pSG5 ERβ or (H) 200 ng of pGL3p-2B7P and 25 ng of pSG5 ERβ were transfected into the HuH-7 cells. Luciferase activity significantly (p < 0.05 One-way ANOVA) different from DMSO is indicated by an asterisk.
We then introduced two point mutations in the ERE to create pGL3-2B6 ERE mut to determine the role of the ERE in the CYP2B6 promoter at mediating the ERα-dependent induction of CYP2B6. We also created a promoter truncation, pGL3-2B6 ΔERE where the putative ERE was removed. pGL3-2B6 ERE mut and pGL3-2B6 ΔERE were transiently transfected with ERα. The E2-induced luciferase activity was not observed in cells transfected with pGL3-2B6 ERE mut and pGL3-2B6 ΔERE (Figure 15 A). In agreement with these findings, the introduction of two mutations, E203A and G204A, in the ERα DNA binding domain (DBD) which prevented ERα-dependent regulation of ERE-mediated responses (Jakacka et al., 2001), reduced the ERα-dependent regulation of pGL3-2B6 (Figure 15 B). Western blots showed that wild-type ERα and DBD mutant were expressed at similar levels, indicating that differences in reporter gene expression were not due to reduced protein levels (Figure 15 C).

Figure 15 ERα-dependent regulation of pGL3-2B6 occurs through the ERE site.

(A) HuH-7 cells were transfected with 200 ng of pGL3-2B6 ERE, pGL3-2B6 ERE mut or pGL3-2B6 ΔERE and 50 ng of pSG5 ERα for 24 h. Cells were treated with either 0.1% DMSO or 10 nM E2 for 24 h prior to luminescence detection. Results shown are means ± S.E.M. for five independent experiments. Luciferase activity that is statistically significant (p < 0.05 One-way ANOVA) from DMSO pGL3-2B6 is indicated by an asterisk. (B) HuH-7 cells were plated in full serum and transfected with 200 ng of pGL3-2B6 ERE and 100 ng of either pSG5 ERα or pSG5 ERαDBD. Results shown are means ± S.E.M. for three independent experiments. Luciferase activity that is statistically significant (p < 0.05 One-way ANOVA) from wild-type ERα is indicated by an asterisk. (C) Western blot analysis shows that the wild-type and the mutant receptors were expressed at similar levels.
**ERα regulates CYP2B6 expression in an E2-dependent manner in T-47D cells**

To investigate the effect of E2 and the ER antagonist ICI 182,780 on CYP2B6 mRNA expression, we isolated RNA from T-47D cells treated with E2 alone, ICI 182,780 alone, or in combination. CYP2B6 mRNA levels were significantly increased in cells treated with 10 nM E2 for 6 h (Figure 16 A) and 24 h. Co-treatment with 100 nM ICI 182,780 effectively reversed the ERα-mediated increase in CYP2B6 mRNA. ICI 182,780 treatment alone had no significant effect on CYP2B6 mRNA expression. We did not observe any increases in CYP2B7P mRNA expression level. Western blot analysis also revealed a modest E2-dependent increase in CYP2B6 protein expression at 24 h (Figure 16 B). ChIP assays showed an E2-dependent increase in recruitment of ERα to the ERE in the 5′ regulatory region of *CYP2B6* at 1 h; however, detectable ERα occupancy was evident in DMSO samples (Figure 16 C). ERα recruitment returned to basal level after 4 h of E2 treatment (Figure 16 C). Although the inhibitory effect of ICI 182,780 on ERα recruitment to the 5′ regulatory region of CYP2B6 was not apparent after 1 h of treatment, ICI 182,780 significantly reduced E2-dependent recruitment of ERα after 4 h (Figure 16 C). RNAi-mediated knockdown of ERα using two different siRNAs targeting ERα resulted in an approximately 30% reduction in CYP2B6 mRNA expression levels (Figure 17 A). This finding demonstrates that ERα plays a role in regulating CYP2B6.
Figure 16 E2-dependent regulation of CYP2B6 expression in T-47D cells. T-47D cells were plated in DCC-FBS containing medium for 72 h before treatment.

(A) After 6 h treatment with 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780, RNA was isolated and CYP2B6 expression level was analyzed by qPCR as described in Materials and Methods. Expression level significantly (p < 0.05 One-way ANOVA) different from DMSO is indicated by an asterisk. Results shown are means ± S.E.M. for three independent experiments. (B) T-47D cells were treated with 0.1% DMSO, 10 nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780 for 24 h before western analysis. Images shown are representative of two independent experiments. (C) ChIP assays were performed in T-47D cells treated with 0.1% DMSO, 10nM E2, 100 nM ICI 182,780, or 10 nM E2 + 100 nM ICI 182,780 for the time points indicated. Results shown are means ± S.E.M. for three independent experiments. Recruitment levels are presented as a percentage of a 5% total chromatin input. Recruitment level significantly (p < 0.05 One-way ANOVA) different from time-matched DMSO is indicated by an asterisk.
Figure 17 RNAi-mediated knockdown of ERα in T-47D cells.

(A) T-47D cells were transfected with either siRNA targeting luciferase (siLuc) or two different siRNA targeting ERα (siERα-11 or siERα-14) for 48 h. RNA was isolated and CYP2B6 and ERα expression levels were analyzed by qPCR as described in Materials and Methods. CYP2B6 mRNA levels were normalized to non-transfected T-47D cells (control), which was set to 1. Statistical significance (p < 0.05 Student’s t-test) compared to siLuc is indicated by an asterisk. Results shown represent the means ± S.E.M. for three independent experiments. (B) Western blot demonstrating significant ERα knockdown.

Differential recruitment of ERα and coactivators is associated with altered expression of TFF1 and CYP2B6 in MCF-7 and T-47D cells

Surprisingly the estrogen responsive TFF1 gene was not identified as an ERα target in our ChIP-chip study in T-47D cells. To investigate these discrepancies between our study and previous studies (Carroll et al., 2006; Kwon et al., 2007), we evaluated the regulation of both of these genes in T-47D and MCF-7 cells. In agreement with another report (Tozlu et al., 2006), we observed increased levels of CYP2B6 mRNA expression in T-47D compared to MCF-7 cells (Figure 18 A). Moreover, T-47D cells exhibited an E2-dependent increase in CYP2B6 mRNA expression that was not observed in MCF-7 cells. Although E2-induced a two-fold increase in TFF1 mRNA levels in both cell lines, the basal level of TFF1 mRNA was three orders of magnitude higher in MCF-7 cells compared to T-47D cells (Figure 18 B). Similar mRNA induction levels of GREB1 were observed in both T-47D and MCF-7 cells (Figure 18 C). ChIP assays revealed increased E2-dependent recruitment of ERα and nuclear
coactivator 3 (NCoA3/SRC3/pCIP/AIB1) to the TFF1 promoter region in MCF-7 cells but not in T-47D cells (Figure 19 A and D). In contrast, E2-dependent increases in ERα and NCoA3 recruitment to the CYP2B6 ERE region were observed in T-47D cells but not in MCF-7 cells (Figure 19 B and E). The recruitment profiles for ERα and NCoA3 to the estrogen responsive GREB1 Enh3 region (Carroll et al., 2006) were similar between T-47D and MCF-7 cells (Figure 19 C and F); although the magnitude of this recruitment was lower in T-47D cells.

Figure 18 Differential expression of TFF1 and CYP2B6 in T-47D and MCF-7 after E2 stimulation.

T-47D and MCF-7 cells were plated in DCC-FBS containing medium for 72 h before treatment. After 6 h treatment with 0.1% DMSO or 10 nM E2, RNA was isolated and the expression of (A) CYP2B6, (B) TFF1, and (C) GREB1 was analyzed by qPCR as described in Materials and Methods. Statistical significance (p < 0.05 Student’s t-test) between treatment groups from the same cell line is indicated by an asterisk. Statistical significance (p < 0.05 Student’s t-test) between cell lines with the same treatment is indicated by a dagger. Results shown are means ± S.E.M. for three independent experiments.
T-47D and MCF-7 cells were plated in DCC-FBS containing medium for 72 h before treatment. ChIP assays were then performed on cells treated with 0.1% DMSO or 10 nM E2 for 1 h. Fig. 9A-C shows ERα recruitment to TFF1, CYP2B6 promoter ERE, and GREB1 Enh 3 respectively. Fig. 9D-F shows NCoA3 recruitment to the three regions. Results shown are means ± S.E.M. for three independent experiments. Recruitment levels are presented as a percentage of a 5% total chromatin input. Recruitment level significantly (p < 0.05 Student’s t-test) different from cell line-matched DMSO is indicated by an asterisk.
Discussion

The regulation of CYP2B6 is important in drug metabolism and xenochemical toxicity (Hodgson and Rose, 2007). Most studies on CYP2B6 regulation have focused on the transcriptional induction mediated by xenobiotic, where the main focus of the studies was the transcriptional regulation of CYP2B enzymes by CAR (Wang and Tompkins, 2008). However, CAR does not operate alone in the transactivation of P450 genes, but rather works in concert with several transcription factors, nuclear receptors, and coactivators to regulate signaling pathways that regulate lipids, bile acids, and hormone homeostasis (Pascussi et al., 2008). Moreover, there are few reports assessing the regulation of CYP2B6 in extrahepatic tissues.

In this study, we demonstrate ER-dependent regulation of CYP2B6 in different human cancer cell lines. Because we detected ERα binding sites in the upstream regulatory region of CYP2B6 gene using ChIP-chip, we characterized the ER-dependent regulation of CYP2B6 gene. QPCR and Western blots revealed that the CYP2B6 mRNA levels were increased in an ER-dependent manner. After analysis of truncated promoters and mutant construct reporters for the gene, we found that ERs increased CYP2B6 promoter activity through an ERE site in the CYP2B6 upstream regulatory region in that gene. ChIP assays confirmed the E2-dependent recruitment of ERα to the 5′-flanking region of CYP2B6 and a distal estrogen responsive region upstream of CYP2B7P; although no changes in CYP2B7P mRNA levels were observed. A significant level of ERα occupancy to both the 5′-regulatory regions of CYP2B6 and CYP2B7P were apparent in the absence of E2. Previous studies have also reported significant ERα binding to different E2-responsive target promoters in the absence of ligand. The high level of basal binding of ERα is unclear, but it might be due to very low levels of estrogens or growth factors present in our estrogen reduced serum-containing medium. In support of this notion, prolonged treated with ICI 182,780, an anti-estrogen that promotes proteolytic degradation of ERα, reduced the level of ERα occupancy below basal levels at both CYP2B6 and CYP2B7P.
Previous studies have reported that high doses of E2 (10 μM) activate mouse CAR, but not human CAR assayed under the same experimental conditions (Kawamoto et al., 2000). The authors concluded that the E2-dependent increase in Cyp2b10 protein levels was independent of ER, since the potent synthetic ER agonist, diethylstilbestrol had no effect. However, this might not be the case for the regulation of CYP2B6. Here we show that physiological levels of E2 induce CYP2B6 mRNA and protein levels in an ERα-dependent manner, but also in a cell line selective manner. The ER action was mediated through an ERE located at -1669 to -1657 bp upstream of the CYP2B6 promoter and 40 bp downstream from the PBREM. The ERE site in human CYP2B6 is not conserved in mouse Cyp2b10, which may explain that the reported E2-dependent changes in Cyp2b10 expression levels might be ER-independent in mice. The proximity of the ERE and PBREM sites suggests crosstalk between CAR and ERα at the CYP2B6 promoter may occur in tissues where both receptors are expressed. Ongoing reporter gene assays revealed that ERα potentiates CAR-mediated regulation of CYP2B6 expression in HuH-7 hepatoma cells (Raymond Lo and Jason Matthews unpublished results). Activating transcription factor (ATF) 5 and members of the CCAAT/enhancer-binding protein potentiate CAR-dependent regulation of CYP2B6 in human hepatocytes and hepatoma cells (Pascual et al., 2008). ERα is highly expressed in liver tissue, and has important regulatory roles in hepatic signaling (Leong et al., 2004a), thus ER might serve as another hepatic regulator of CYP2B6 expression. Furthermore, given that ERs are expressed in several extrahepatic tissues, it is possible that ERs might serve as an extrahepatic regulator of CYP2B6 levels. Further investigation is needed to understand the specific transcriptional environment that determines the role of ERs in tissue-specific regulation of CYP2B6.

CYP2B6 metabolizes a wide range of xenobiotic and endogenous compounds and is an important regulator of hormone homeostasis (Gonzalez, 1991). CYP2B6 catalyzes the hydroxylation of testosterone (Imaoka et al., 1996; Waxman et al., 1988) and thus indirectly influences E2 levels. Since, ERs are also known to regulate the expression of CYP1A1 and CYP1B1, both of which metabolize endogenous estrogens (Frasor et al., 2004; Kininis et al., 2007; Tsuchiya et al., 2004; Tsuchiya et al., 2005), the ER-dependent regulation of CYPs may be part of a regulatory mechanism to control estrogen and/or other hormone levels.
A comparison of the ChIP-chip data from T-47D cells with similar studies using MCF-7 cells (Carroll et al., 2006; Kwon et al., 2007) revealed that only about a third of the identified ERα-bound regions overlapped with ERα-bound regions following E2-treatment of MCF-7 cells (Carroll et al., 2006). The cell-line specific differences in ERα-bound regions might be due to differences in assay conditions, array platforms and data analysis strategies between the studies (Carroll et al., 2006; Kwon et al., 2007). In terms of CYP2B6, E2-dependent regulation of this gene was evident in T-47D but not MCF-7 cells. Conversely, the recruitment level of ERα to the promoter region of TFF1 was not detectable in T-47D cells after 1 h of E2 treatment. These differences were due to cell line-specific differences in E2-dependent occupancy of ERα and NCoA3. Despite the lack of E2-induced recruitment of ERα to TFF1 in T-47D cells, we observed a 2-fold increase in TFF1 mRNA levels. This may be due to differences in sensitivity between ChIP assays and mRNA expression determination by qPCR. Nevertheless, the identification of different ERα-bound regions between the two cell lines highlights the importance of studying ERα activity in multiple breast cancer cell lines to obtain a complete understanding of ERα-dependent transcription.

In the studies reported here, we have established that ERs regulate CYP2B6 expression through direct binding to an ERE located in the CYP2B6 promoter, but in a breast cancer cell line selective manner. Our data add ERs to the growing list of transcription factors involved in the regulation of CYP2B6 expression. We have also demonstrated differential ERα-mediated gene expression in two commonly used ERα-positive breast cancer cell lines, indicating that it is necessary to use multiple cancerous, immortalized, and/or primary cell lines to gain a more accurate understanding of ER-mediated transcription.
Significance of Study

Whereas all previous genomic analyses of estrogen receptor binding sites were conducted in steroid deprived human MCF-7 breast cancer cells treated with E2, our study was the first to determine ERα binding sites in T-47D breast cancer cells constitutively exposed to serum level of estrogen. Under our experimental conditions, we identified substantial ERα recruitment to two regulatory regions in the CYP2B locus, which corroborated previous reports of E2-induced CYP2B6 expression in breast tissues and E2-induced CYP2B1 expression in rat livers. Analysis of the two ERα binding sites indicated that E2 dependent expression of CYP2B6 was mediated through ERα binding to two imperfect EREs in proximity of the PBREM. Comparison between MCF-7 and T-47D cells revealed cell-line specific differences, which might explain why the two ERα binding sites were not identified in previous genomic analyses of ERα binding sites in MCF-7. Taken together from a molecular endocrinological point of view, our study demonstrates cell-line specific differences in estrogen response. This highlights the need to use multiple cell lines to get a better representation of ERα-dependent transcription in mammary cells.

From a pharmacological point of view, this study highlights the importance of estrogen receptor in regulating xenobiotic metabolism in addition to its well defined role in endocrine and hormonal regulation. Recently, estrogen receptors have been demonstrated to directly regulate a wide range of enzymes implicated in Phase I and Phase II metabolism, including CYP2B6, CYP2A6, CYP1B1, NADPH quinone oxidoreductase 1 (NQO1) and glutathione-S-transferase (GST). Deregulation of Phase I and Phase II metabolism by estrogen may offset the balance between toxic and non-toxic metabolites leading to a cellular environment that might be favorable for the development and/or progression of breast cancer.
Chapter 3 Identification of Aryl Hydrocarbon Receptor Binding Targets in Mouse Hepatic Tissue Treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin

Raymond Lo, Agnes L. Forgacs, Edward Dere, Trine Celius, Laura MacPherson, Patricia Harper, Timothy Zacharewski, Jason Matthews

Based on Toxicology and Applied Pharmacology, 2011

All experiments were conducted by Raymond Lo with the exception of the chromatin immunoprecipitation-on-microarray chip experiments, which were conducted by Jason Matthews. Animal husbandry and tissue collection were performed by Laura MacPherson, Trine Celius, and Patricia Harper. Data analyses were performed by Raymond Lo, Edward Dere, Agnes L. Forgacs and Jason Matthews. The study was designed by Jason Matthews. Jason Matthews and Timothy Zacharewski are collaborators on using ChIP-chip to assess the biological and toxicological effects of dioxin exposure in animal models. The final manuscript was written by Raymond Lo and Jason Matthews.

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Abstract

Genome-wide, promoter-focused ChIP-chip analysis of hepatic aryl hydrocarbon receptor (AHR) binding sites was conducted in 8-week old female C57BL/6 treated with 30 \( \mu \text{g/kg/body weight} \) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 2h and 24h. These studies identified 1,642 and 508 AHR-bound regions at 2h and 24h, respectively. A total of 430 AHR-bound regions were common between the two time points, corresponding to 403 unique genes. Comparison with previous AHR ChIP-chip studies in mouse hepatoma cells revealed that only 62 of the putative target genes overlapped with the 2h AHR-bound regions in vivo. Transcription factor binding site analysis revealed an over-representation of aryl hydrocarbon response elements (AHREs) in AHR-bound regions with 53% (2h) and 68% (24h) of them containing at least one AHRE. In addition to AHREs, E2f-Myc activator motifs previously implicated in AHR function, as well as a number of other motifs, including Sp1, nuclear receptor subfamily 2 factor, and early growth response factor motifs were also identified. Expression microarray studies identified 133 unique genes differentially regulated after 4h treatment with TCDD. Of which, 39 were identified as AHR-bound genes at 2h. Ingenuity Pathway Analysis on the 39 AHR-bound TCDD responsive genes identified potential perturbation in biological processes such as lipid metabolism, drug metabolism, and endocrine system development as a result of TCDD-mediated AHR activation. Our findings identify direct AHR target genes in vivo, highlight in vitro and in vivo differences in AHR signaling and show that AHR recruitment does not necessarily result in changes in target gene expression.
Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated receptor and member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) transcription factor family that regulates the adaptive metabolic response to planar aromatic hydrocarbons. Upon agonist binding, the AHR translocates to the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The heterodimer complex binds to genomic enhancer elements known as aryl hydrocarbon response elements (AHREs; 5’-GCG TG-3’) located within the regulatory regions targeted genes, including a battery of phase I and phase II xenobiotic metabolizing enzymes, such as cytochrome P450s 1a1 and 1b1 (Cyp1a1 and Cyp1b1), UDP glucuronosyltransferase 1a1 (Ugt1a1), and NADPH dehydrogenase 1 (Nqo1) (Hankinson, 1995).

The AHR also mediates the toxic responses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a ubiquitous environmental contaminant that elicits diverse species-specific effects, including tumor promotion, teratogenesis, hepatotoxicity, immunotoxicity and modulation of endocrine systems (Birnbaum, 1994a, b; Poland and Knutson, 1982). Many of these effects are the result of alterations in AHR-mediated gene expression (Hankinson, 1995). Ahr-null mice are resistant to many of the responses typically observed following treatment with TCDD and related compounds (Fernandez-Salgueiro et al., 1996). More recently, AHR has been shown to have an important developmental role in vascular remodeling of the liver, where it is essential for the proper postnatal closure of the ductus venosus (Harstad et al., 2006; Lahvis et al., 2005). Studies using recombinant animal models suggest that the adaptive, toxic and developmental pathways share similar upstream signaling events including AHR activation, formation of the AHR/ARNT heterodimer, binding of the heterodimer complex to AHREs and changes in target gene expression (Bunger et al., 2008; Bunger et al., 2003; Schmidt et al., 1996). However, recent studies provide evidence that AHR-dependent suppression of inflammation occurs through an AHRE-independent mechanism (Flaveny et al., 2010). As with other transcription factors, ligand-induced recruitment of coregulator proteins (coactivators and corepressors) is also critical for AHR-mediated transcription (Hankinson, 2005). Variations in coactivator expression may underlie tissue-specific differences in ligand-sensitivity and target gene expression (Hankinson,
Although the AHR signaling pathway is well understood, the gene regulatory pathways responsible for TCDD-mediated toxicity remain largely unknown and further investigation is needed to assess the potential risks to humans and other species.

AHR-DNA interactions have been primarily studied for the regulation of \textit{CYP1A1} (Hankinson, 1995; Whitlock, 1999), but recent chromatin immunoprecipitation combined with microarrays (ChIP-chip) studies have extended our knowledge of AHR-DNA interactions by examining AHR enrichment at promoter regions using \textit{in vitro} human and mouse cell models (Ahmed et al., 2009; Pansoy et al., 2010; Sartor et al., 2009). These studies identified a number of novel genomic regions bound by AHR and also revealed that AHR occupies several genomic regions in the absence of exogenous ligand activation. Since these studies were done in immortalized cell lines they may not reflect changes that occur \textit{in vivo}. Large variations in AHR-regulated gene expression between mouse hepatoma cells and mouse liver have been reported (Dere et al., 2006).

Since the liver is a primary target tissue for TCDD-mediated toxicity, we used ChIP-chip to study the hepatic binding of AHR to genomic regions in C57BL/6 mice and compared the AHR-bound regions with gene expression arrays to identify AHR-regulated gene networks important in TCDD-mediated toxicity. Ingenuity Pathway Analysis (IPA; www.ingenuity.com) was then used to identify enriched pathways that were significantly perturbed by TCDD. Our study identifies direct AHR target genes \textit{in vivo}, and highlights the discrepancy between AHR action \textit{in vivo} and \textit{in vitro}. 
Materials and Methods

Animal Husbandry

Mature intact 8-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed at the Hospital for Sick Children (HSC; Toronto, ON) under conditions described previously (Choi et al., 2006). Animals were injected once intraperitoneally with 30 μg/kg bodyweight of TCDD (Wellington Laboratories; Guelph, ON) in corn oil (Sigma-Aldrich; Oakville, ON) or with corn oil alone and were sacrificed 0.5, 2, 4, and 24h post dose. Livers were immediately removed and frozen at -80°C for AHR binding analysis using chromatin immunoprecipitation. Care and treatment of the animals were done according to the guidelines set by the Canadian Council on Animal Care and approved by HSC Animal Care Committee.

Cell Culture

Hepa1c1c7 mouse hepatoma cells were maintained in Minimum Essential Medium Alpha (MEMalpha; Invitrogen Corp., Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Wisent, St.-Bruno, QC) and 1% penicillin-streptomycin mix (PEST; Wisent) at 37°C and 5% CO₂. Cells were plated at a density of approximately 1 million cells per 10 cm dish and were treated with either 0.1% DMSO or 10 nM TCDD for the time indicated.

Chromatin Immunoprecipitation (ChIP) and ChIP-chip experiments

ChIP assays were performed as previously described (Pansoy et al., 2010) with the following changes. Approximately 100 mg of frozen mouse liver was homogenized in 1% formaldehyde and incubated for 10 min at room temperature. After centrifugation, the pellet was resuspended in 900 μL of TSEI (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate) + 1x Protease Inhibitor Cocktail (Sigma, St. Louis, MO) and sonicated 12 x 10s using a Branson 450 sonifier. Supernatant
was transferred to fresh microcentrifuge tubes and incubated with rabbit IgG (5 μg; Sigma) or anti-AHR (5 μg; SA-210, Enzo Life Sciences Inc, Farmingdale, NY) O/N at 4°C. ChIP samples were washed and the DNA isolated as previously described (Celius et al., 2010). For ChIP-chip experiments, immunoprecipitated DNA was linearly amplified using a whole genome amplification kit according to the manufacturer’s instructions (Sigma). Linearly amplified DNA (7.5 μg) was fragmented by limited DNaseI digestion and hybridized to Affymetrix Mouse Promoter Tiling Arrays 1.0R (Affymetrix, Santa Clara, CA) as previously described (Pansoy et al., 2010). The hybridization and washing steps were performed according to the manufacturer’s protocol at the Centre for Applied Genomics (Toronto, Canada). Data were normalized and analyzed using CisGenome and mapped against mouse genome version mm9 (Ji et al., 2008). Enriched peaks were determined by comparing quadruplet samples of AHRTCDD to triplicate IgGTCDD using a moving average approach with default settings in TileMap v2 (Ji and Wong, 2005). ChIP-chip results were confirmed by QPCR. Recruitment level is presented as a percentage of total DNA input.

**Transcription factor binding motif analysis**

Transcription factor binding site analysis was done using the commercially available software MatInspector and RegionMiner from Genomatix (www.genomatix.de). The AHR$_{2h}$ regions identified by ChIP-chip were compared against 2,000 randomly selected promoter sequences compiled from 7.5 kb upstream to 2.5 kb downstream of known transcription start sites. Enriched transcription factor binding motifs were determined based on their Z-scores and we filtered out any transcription factor binding sites that did not meet the cutoff of at least 3 standard deviations. To identify transcription factors that might work in concert with AHR to modulate AHR transcription, we also determined the over-representation of transcription factor binding motifs located within 10 to 50 bp from putative AHREs in the AHR$_{2h}$ regions. Information on the definitions of the family matrices discussed herein is available on the Genomatix web page (www.genomatix.de).
**RNA extraction, expression array analysis, cDNA synthesis**

Approximately 50mg of frozen mouse liver was homogenized in the lysis buffer provided in the Aurum Total RNA Mini Kit (Bio-Rad, Toronto, ON). Total RNA was extracted according to manufacturer’s protocol. Extracted RNA was processed, labeled, and hybridized to the GeneChip Mouse Exon 1.0 ST Array (Affymetrix) at the Centre for Applied Genomics (Toronto, ON). Statistical significance was determined by ANOVA using Partek Genomics Suite (Partek, St. Louis, MO). A list of differentially regulated genes was generated using a cutoff of absolute fold change $\geq 1.5$ and $p$-value $\leq 0.01$.

The expression levels of AHR-bound genes from our ChIP-on-chip studies were quantified using QPCR. Briefly, 500ng of total RNA was reverse transcribed with random hexamer using Super Script Reverse Transcriptase II (SSRTII, Invitrogen).

**Function and Network Analysis**

Ingenuity Pathway Analysis (www.ingenuity.com) was used to examine the biological functions/diseases and potential interactions among all of the AHR-regulated target genes identified in our studies.

**Statistical Analyses**

All results are expressed as means $\pm$ standard deviations. Statistical analysis was performed using the Student's two tailed $t$-tests and assessed at $p < 0.05$. The significance values of biological functions/diseases identified in the IPA studies were calculated using the right-tailed Fisher’s exact test.
Results

Kinetics of AHR recruitment in mouse liver and in Hepa1c1c7 mouse hepatoma cells

To determine the time point of maximal AHR and ARNT recruitment to the Cyp1a1 AHRE containing enhancer region we performed ChIP assays in vivo using C57BL/6 mice that were treated with a single intraperitoneal injection with 30 μg/kg/bw TCDD for 0.5h, 2h, 4h and 24h (Figure 20 A and C). Significant TCDD-dependent AHR and ARNT recruitment to Cyp1a1 AHRE was detected as early as 0.5h with higher recruitment levels observed after 2h, 4h and 24h treatment. Based on these data we chose the 2h and 24h time points for ChIP-chip analysis to identify AHR-bound regions in vivo. Comparative time course analyses were done in Hepa1c1c7 mouse hepatoma cells, a cell line commonly used to study AHR-dependent transcription. As expected, these experiments revealed a different kinetic recruitment profile of AHR and ARNT compared to that observed in vivo. Maximal AHR and ARNT recruitment was observed after 30 min of treatment of Hepa1c1c7 cells with 10 nM of TCDD (Figure 20 B and D). AHR and ARNT recruitment decreased to submaximal levels at 4h and 24h TCDD treatment, which was in contrast to the relatively stable AHR and ARNT occupancy at the Cyp1a1 AHRE in vivo after 4h and 24h TCDD treatment. However, differences in the relative uptake and cellular accumulation of TCDD between normal tissue and the immortalized cell line, Hepa1c1c7, may also contribute to the observed temporal differences in AHR recruitment to Cyp1a1.
ChIP assays were performed to evaluate AHR and ARNT temporal recruitment profile in liver or in Hepa1c1c7 hepatoma cells exposed to TCDD. (A & C) C57BL/6 mice were treated intraperitoneally with either corn oil or 30 μg/kg/bw TCDD for 0.5, 2, 4, and 24 h. ChIPs assays were performed on isolated liver extracts as described in Materials and Methods using anti-AHR (A) or anti-ARNT (C) antibodies. (B & D) Hepa1c1c7 cells were treated with either DMSO or 10 nM TCDD for 0.5, 4, and 24 h. ChIP assays were performed as described in Materials and Methods using anti-AHR (B) or anti-ARNT (D) antibodies. Results represent the mean values of at least two independent replicates for hepalc1c7 cells and four independent replicates for liver samples. Statistical significance (p < 0.05, Student’s t-test) compared to time-matched vehicle control is indicated with an asterisk.
Identification of AHR-bound regions in mouse liver

To identify TCDD-induced AHR-bound regions in mouse liver, we performed ChIP-chip assays on liver extracts isolated from 8-week old intact female C57BL/6 mice treated with 30 μg/kg/bw TCDD for 2h and 24h. A total of 3,114 and 2,195 AHR-bound regions were identified at 2h and 24h, respectively. Using a false detection rate cut-off (FDR) of 1%, we identified a final set of 1,642 (AHR\(_{2h}\)) and 508 (AHR\(_{24h}\)) regions. A total of 430 AHR-bound regions (26% of the 2h and 85% of the 24h regions), herein referred to as AHR\(_{overlap}\), overlapped with at least 50% sequence identity between both time points (Figure 21 A). Specific AHR regions are referred to as AHR\(_{2h\_number}\) or AHR\(_{24h\_number}\) where the number indicates the relative rank of AHR enrichment in the region within each of the respective analyses. The enriched regions were determined by comparing AHR-bound regions to IgG, thus AHR enrichment may or may not be dependent on TCDD treatment. Since each of the isolated regions could be labeled with a target gene, we also assessed the overlap between the AHR\(_{2h}\) regions and the AHR\(_{24h}\) regions in terms of target genes. We found that 30% (403) of the 2h and 87% of the 24h putative target genes were common between the AHR\(_{2h}\) (1,339) and AHR\(_{24h}\) (461) data sets.

![Figure 21 Venn diagram of the overlap between (A) AHR-bound regions at 2h and 24h, and (B) genes associated with AHR-bound regions in our study at 2h and those identified by Sartor and colleagues (Sartor et al., 2009).](image)

The total number of regions/genes in each data set is shown in parentheses.

Since the majority of the putative target genes identified in the AHR\(_{24h}\) data were present in the AHR\(_{2h}\) data, we focused on the 1,339 putative target genes that corresponded
to the AHR\textsubscript{2h} regions and compared them to a previously published ChIP-chip data set from mouse Hepa1c1c7 mouse hepatoma cells (Ahmed et al., 2009; Sartor et al., 2009). The Hepa1c1c7 cells were treated with 5 nM TCDD for 1.5h and hybridized to the same Affymetrix GeneChip Mouse Promoter 1.0R Array platform used in our study (Sartor et al., 2009). Of the 750 TCDD-induced and AHR-bound regions or 747 target genes reported in Hepa1c1c7 cell study, only 62 unique genes were present in our AHR\textsubscript{2h} data set (Figure 21 B). Given the known species differences in AHR responsiveness (Flaveny et al., 2010), we also compared the AHR\textsubscript{2h} binding sites in our mouse hepatic model to our previous study in which we identified AHR bound regions following 1h treatment of using T-47D human breast cancer cells (Ahmed et al., 2009). Of the 383 AHR-bound genes identified in T-47D cells, only 40 unique AHR-bound genes were common to both species and included many of the classic genes characteristic of the AHR gene-battery. These findings highlight the differences of AHR signaling \textit{in vivo} and \textit{in vitro}, and support the relatively low degree of overlap of TCDD-responsive genes regulated by human AHR compared to those regulated by mouse AHR (Flaveny et al., 2010); (Dere et al., 2006).

**Transcription Factor Binding Motif Analysis**

To determine the number of AHR-bound regions that contained at least one core AHRE, we searched all of the AHR\textsubscript{2h} and AHR\textsubscript{24h} regions for AHREs using MatInspector (www.genomatix.de). This analysis revealed that 53\% (875) of the AHR\textsubscript{2h}, 68\% (345) of the AHR\textsubscript{24h} and 72\% (310) of the AHR\textsubscript{overlap} regions contained at least one core AHRE (5\textasciitilde-GCGTG-3\textasciitilde). We then searched the AHR\textsubscript{2h} regions for the over-representation of other transcription factor binding motifs using RegionMiner (Genomatix) (Table 2). We focused on the AHR\textsubscript{2h} regions since the majority of the AHR\textsubscript{24h} regions were present in the AHR\textsubscript{2h} data set. This screen identified AHREs, E2f-Myc activator (E2F) and S\textit{p}1 motifs as previously implicated in AHR function (Marlowe et al., 2004); (Tsuchiya et al., 2003), as well as a number of other motifs, including hypoxia inducible factor (HIF), nuclear receptor subfamily 2 factor (NR2F), and early growth response factor (EGRF) motifs. Interestingly, 86\% of AHR\textsubscript{2h} regions contained at least one putative AHRE, E2f-Myc or S\textit{p}1 binding site.
Thus AHR binding at many of the identified regions might be due to AHR tethering to E2F or Sp1 transcription factors.

**Table 2** Significantly over-represented transcription factor family binding motifs in the AHR$_{2h}$ regions$^{a}$ using RegionMiner (Genomatix).

<table>
<thead>
<tr>
<th>TF Module Family</th>
<th>Module Description</th>
<th>2 h AHR bound regions$^1$</th>
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</thead>
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<td></td>
<td># of matches</td>
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<td>E2F-Myc activator</td>
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</table>

$^1$ AHR bound regions with a 1% FDR

To identify other transcription factors that might work in concert with AHR to modulate AHR function we searched the AHR$_{2h}$ regions for enriched transcription factor motifs that were located within 10 to 50 bp of AHREs using RegionMiner (Genomatix). The proximity of an AHRE and Nfe2l2 binding site, known as an antioxidant response element, has been reported to modulate the regulation of murine Nqo1. Mutation of the antioxidant response element prevented the AHR-mediated regulation of Nqo1 (Lin et al., 2011). We found an enrichment of AHREs in close proximity to E2F, Sp1, as well as forkhead box
(FKHD) and NR2F motifs, suggesting that these transcription factors might work as regulatory modules in AHR-dependent gene expression (Table 3).

Table 3 Significantly over-represented transcription factor family binding motifs within 10-50 bp of AHREs in the AHR$_{2h}$ regions$^1$ using RegionMiner (Genomatix).

<table>
<thead>
<tr>
<th>TF Module</th>
<th>TF Module Description associated with AHRE</th>
<th>2 h AHR bound regions$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td># of matches</td>
</tr>
<tr>
<td>AHR_EGRF</td>
<td>EGR/nerve growth factor induced protein C</td>
<td>620</td>
</tr>
<tr>
<td>AHR_E2F</td>
<td>E2F-Myc activator</td>
<td>391</td>
</tr>
<tr>
<td>AHR_NRF1</td>
<td>Nuclear respiratory factor 1</td>
<td>335</td>
</tr>
<tr>
<td>AHR_SP1</td>
<td>GC-box factors SP1/GC</td>
<td>412</td>
</tr>
<tr>
<td>AHR_CREB</td>
<td>cAMP-responsive element binding proteins</td>
<td>446</td>
</tr>
<tr>
<td>AHR_NR2F</td>
<td>Nuclear receptor superfamily 2 factors</td>
<td>431</td>
</tr>
<tr>
<td>AHR_CEBP</td>
<td>Ccaat/Enhancer Binding Protein</td>
<td>199</td>
</tr>
<tr>
<td>AHR_FKHD</td>
<td>Forkhead domain factors</td>
<td>407</td>
</tr>
</tbody>
</table>

$^1$ AHR bound regions with a 1% FDR

ChIP-on-chip confirmation

To confirm the enriched regions identified by ChIP-on-chip, independent ChIP assays were done using a different section of the same liver from the corn oil or TCDD-treated animals. We examined 18 positive AHR$_{2h}$ regions, representing regions with a range of AHR enrichment values as well as regions corresponding to genes of interest to our laboratory (Figure 22). We also included the Cyp1a1 AHRE enhancer region as a positive control in our confirmation since this sequence was absent from the Affymetrix GeneChip Mouse Promoter 1.0R Array (Sartor et al., 2009). We confirmed AHR recruitment to 19/19 regions
that mapped to Cyp1a1 (AHRE cluster), gamma-aminobutyric acid receptor associated protein (Gabarap, AHR2h.1), nuclear factor erythroid derived 2 like 2 (Nfe2l2, AHR2h.4), TCDD-inducible poly(ADP-ribose) polymerase (Tiparp, AHR2h.10), hairy enhancer of split 1 (Hes1, AHR2h.13), Cyp1b1 (AHR2h.14), flavin-containing monooxygenase 3 (Fmo3, AHR2h.53), serpin peptidase inhibitor nexin clade E member 1 (Serpine1 AHR2h.71), forkhead box O3 (Foxo3, AHR2h.123 and AHR2h.270), peroxisome proliferator-activated receptor α (Ppara, AHR2h.187), Cyp1a2 (AHR2h.220), Nqo2 (AHR2h.268), erb-b2 erythroblastic leukemia viral oncogene homolog 3 (Erbb3, AHR2h.311), Jun (AHR2h.425), a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 7 (Adams7, AHR2h.476), prospero homeobox 1 (Prox1, AHR2h.497), Nqo1 (AHR2h.579), and growth arrest and DNA-damage-inducible 45 gamma (Gadd45γ, AHR2h.774). Of the regions confirmed, Gabarap, Foxo3, Ppara, Erbb3, Adams7, Jun and Gadd45γ were AHR-bound genes whose regulation by AHR has not been extensively examined. As shown in Figure 22, AHR was not associated with the majority of the identified regions in the absence of TCDD, with the occupancy of AHR increasing at these regions in the TCDD treated samples. These findings support the predominant ligand-dependent recruitment of AHR to its target genes. A recent study in Hepa1c1c7 cells suggested that AHR occupies genomic regions in the absence of exogenous ligand with treatment of AHR agonists causing a change in the genomic regions bound by AHR (Sartor et al., 2009). We also observed TCDD-independent occupancy at Gabarap (AHR2h.1) and to a lesser extent to Nfe2l2 (AHR2h.4). AHR occupancy at Gabarap (AHR2h.1) was not further increased with TCDD treatment, whereas increased AHR recruitment to Nfe2l2 (AHR2h.4) in the TCDD-treated group was observed (Figure 22). However, AHR occupancy in the absence of TCDD was evident at only a small fraction of the genomic regions analyzed.
Figure 22 Confirmation of AHR recruitment to regions identified by ChIP-chip.

Nineteen AHR-bound regions (including Cyp1a1 AHRE) were chosen to represent a range of enrichment values from our ChIP-chip analysis at 2h. AHR recruitment is shown as a percentage of immunoprecipitated AHR-bound relative to total input chromatin. Results represent the mean values of four independent replicates. Statistical significance (p < 0.01, Student’s t-test) compared to IgG control is indicated with an asterisk. For clarity, IgG controls are not shown and did not vary among the samples.

We then determined AHR enrichment to a subset of 11 of the 19 (including Cyp1a1 AHRE) regions in TCDD-treated Hepa1c1c7 cells. Based on the results from the time course experiments presented in Figure 20 A and B, we compared AHR recruitment in the liver extracts from female mice treated by intraperitoneal injection with TCDD for 2h (Figure 22) and in Hepa1c1c7 cells treated with 10 nM TCDD for 30 min (Figure 23 A). Although AHR recruitment to all regions was confirmed in liver extracts treated with TCDD for 2h (Figure 22), AHR occupancy was only detected at 7 out of the 11 regions in 30 min treated Hepa1c1c7 cells. We then confirmed AHR recruitment to the same set of 11 regions in livers of female mice treated with TCDD for 24h (Figure 23 B) and compared them to Hepa1c1c7 cells treated for 24h with 10 nM TCDD (Figure 23 C). AHR recruitment was confirmed to all 11 regions in the 24h in vivo liver group, but only to 4 out of the 11 regions after 24 h treatment in vitro. These findings highlight the complexities and temporal differences of AHR signaling in vivo and in vitro.
Figure 23 AHR recruitment to 11 regions (including Cyp1a1 AHRE) was examined.

(A) Hepa1c1c7 treated with 10nM TCDD for 0.5h, (B) hepatic tissue from animals treated with 30 μg/kg TCDD for 24h, and (C) Hepa1c1c7 treated with 10nM TCDD for 24h. AHR recruitment is shown as a percentage of immunoprecipitated AHR-bound relative to total input. Results represent the mean values of at least three independent replicates. Statistical significance (p < 0.01, Student’s t-test) compared to vehicle control is indicated with an asterisk.
Identification of TCDD-induced, AHR-regulated genes in mouse liver

To identify TCDD-induced and AHR-bound target genes, we performed gene expression microarray studies on RNA isolated from female mice livers treated with TCDD for 4h. This time point was chosen to maximize the chances of identifying direct AHR target genes. At a cutoff of |fold change| ≥ 1.5 and \( p \leq 0.01 \), we identified 133 unique differentially regulated genes. We found that 94 (71%) of these genes were up-regulated and 39 (29%) were down-regulated. Of the 133 genes, 39 (30%) were identified as AHR-bound genes in our ChIP-chip studies (Figure 24 A and Table 4). Transcription factor binding site analysis using MatInspector (Genomatix) showed that 34 of the 39 AHR-bound TCDD responsive genes contained at least one AHRE, although many of them contained multiple AHR binding sites.

We then compared TCDD-regulated genes identified from microarray analysis with the AHR-bound regions determined from our ChIP-chip studies. We next examined the TCDD-responsiveness of the 18 genes corresponding to the 19 genomic regions (2 regions corresponded to Foxo3) analyzed in Figure 22 using qPCR. We found that 9 of 10 TCDD responsive genes were also identified in the microarray analysis with the exception of Nqo2, which was significant at \( p < 0.05 \). Conversely, Jun was found to be TCDD-responsive by microarray expression analysis, but this could not be confirmed by qPCR. All other regions were not TCDD-responsive by microarray analysis or qPCR. Taken together, these data provide further evidence for the importance of AHRE in AHR-regulated target gene expression and show that AHR occupancy is not always predictive for TCDD-induced gene expression.
### Table 4. List of TCDD-responsive AHR-regulated genes and their corresponding AHR-bound region.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Type</th>
<th>Fold change from the array at 4h</th>
<th>Corresponding AHR region at 2h</th>
<th>AHRE present?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2610301F02Rik</td>
<td>n/a</td>
<td>n/a</td>
<td>1.94</td>
<td>AHR$_{2h,21}$</td>
<td>Yes</td>
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<tr>
<td>A230050P20Rik</td>
<td>n/a</td>
<td>n/a</td>
<td>1.90</td>
<td>AHR$_{2h,767}$</td>
<td>No</td>
</tr>
<tr>
<td>Abcd2</td>
<td>ATP-binding cassette, sub-family D (ALD), member 2</td>
<td>transporter</td>
<td>2.00</td>
<td>AHR$_{2h,94}$</td>
<td>No</td>
</tr>
<tr>
<td>Bmf</td>
<td>Bcl2 modifying factor</td>
<td>other</td>
<td>2.28</td>
<td>AHR$_{2h,279}$</td>
<td>Yes</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>cytochrome P450, family 1, subfamily A, polypeptide 2</td>
<td>enzyme</td>
<td>1.78</td>
<td>AHR$_{2h,220}$</td>
<td>No</td>
</tr>
<tr>
<td>Cyp1b1</td>
<td>cytochrome P450, family 1, subfamily B, polypeptide 1</td>
<td>enzyme</td>
<td>3.21</td>
<td>AHR$_{2h,14}$</td>
<td>Yes</td>
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<tr>
<td>Dapk2</td>
<td>death-associated protein kinase 2</td>
<td>kinase</td>
<td>2.23</td>
<td>AHR$<em>{2h,234}$, AHR$</em>{2h,431}$</td>
<td>Yes</td>
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<tr>
<td>Edc3</td>
<td>enhancer of mRNA decapping 3 homolog (S. cerevisiae)</td>
<td>other</td>
<td>2.12</td>
<td>AHR$_{2h,66}$</td>
<td>Yes</td>
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<td>Eml4</td>
<td>echinoderm microtubule associated protein like 4</td>
<td>other</td>
<td>2.60</td>
<td>AHR$_{2h,633}$</td>
<td>Yes</td>
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<tr>
<td>Fbxw9</td>
<td>F-box and WD repeat domain containing 9</td>
<td>other</td>
<td>2.04</td>
<td>AHR$<em>{2h,287}$, AHR$</em>{2h,591}$</td>
<td>Yes</td>
</tr>
<tr>
<td>Gan</td>
<td>gigaxxonin</td>
<td>other</td>
<td>-1.52</td>
<td>AHR$<em>{2h,771}$, AHR$</em>{2h,1590}$</td>
<td>Yes</td>
</tr>
<tr>
<td>Gata6</td>
<td>GATA binding protein 6</td>
<td>transcription regulator</td>
<td>-1.61</td>
<td>AHR$_{2h,448}$</td>
<td>Yes</td>
</tr>
<tr>
<td>Gclc</td>
<td>glutamate-cysteine ligase, catalytic subunit</td>
<td>enzyme</td>
<td>1.99</td>
<td>AHR$<em>{2h,1386}$, AHR$</em>{2h,334}$</td>
<td>Yes</td>
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<tr>
<td>Hes1</td>
<td>hairy and enhancer of split 1, (Drosophila)</td>
<td>transcription regulator</td>
<td>1.55</td>
<td>AHR$<em>{2h,13}$, AHR$</em>{2h,961}$</td>
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<tr>
<td>Hspa4l</td>
<td>heat shock 70kDa</td>
<td>other</td>
<td>1.50</td>
<td>AHR$_{2h,472}$</td>
<td>Yes</td>
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<tr>
<td>Protein Name</td>
<td>Description</td>
<td>Function</td>
<td>Log2 Fold Change</td>
<td>AHR2h Accessions</td>
<td>Regulated</td>
</tr>
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<td>---------------------</td>
<td>------------------</td>
<td>-----------------</td>
<td>-----------</td>
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<tr>
<td>Htatip2</td>
<td>HIV-1 Tat interactive protein 2, 30kDa</td>
<td>transcription regulator</td>
<td>3.57</td>
<td>AHR2h_135, AHR2h_451</td>
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<td>Ier3</td>
<td>immediate early response 3</td>
<td>other</td>
<td>1.98</td>
<td>AHR2h_1316</td>
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<td>Josd1</td>
<td>Josephin domain containing 1</td>
<td>other</td>
<td>1.65</td>
<td>AHR2h_272, AHR2h_372</td>
<td>Yes</td>
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<tr>
<td>Jun</td>
<td>jun proto-oncogene</td>
<td>transcription regulator</td>
<td>-1.74</td>
<td>AHR2h_425, AHR2h_760, AHR2h_882</td>
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<td>Klb</td>
<td>klotho beta</td>
<td>enzyme</td>
<td>1.71</td>
<td>AHR2h_787</td>
<td>Yes</td>
</tr>
<tr>
<td>Lmo7</td>
<td>LIM domain 7</td>
<td>enzyme</td>
<td>2.10</td>
<td>AHR2h_1056, AHR2h_78</td>
<td>Yes</td>
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<td>Lrp4</td>
<td>low density lipoprotein receptor-related protein 4</td>
<td>other</td>
<td>2.08</td>
<td>AHR2h_1086, AHR2h_842</td>
<td>Yes</td>
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<tr>
<td>Meig1</td>
<td>meiosis expressed gene 1 homolog (mouse)</td>
<td>other</td>
<td>3.02</td>
<td>AHR2h_776</td>
<td>Yes</td>
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<td>Nags</td>
<td>N-acetylglutamate synthase</td>
<td>enzyme</td>
<td>1.81</td>
<td>AHR2h_7, AHR2h_587</td>
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<td>Nfe2l2</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
<td>transcription regulator</td>
<td>2.44</td>
<td>AHR2h_4</td>
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<td>Nup155</td>
<td>nucleoporin 155kDa</td>
<td>transporter</td>
<td>2.18</td>
<td>AHR2h_25</td>
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<td>Palmd</td>
<td>palmdelphin</td>
<td>other</td>
<td>-1.60</td>
<td>AHR2h_1611</td>
<td>Yes</td>
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<td>Pml</td>
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<td>transcription regulator</td>
<td>1.59</td>
<td>AHR2h_1417</td>
<td>Yes</td>
</tr>
<tr>
<td>Pmm1</td>
<td>phosphomannomutase 1</td>
<td>enzyme</td>
<td>1.54</td>
<td>AHR2h_1473</td>
<td>No</td>
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<td>Saa1</td>
<td>serum amyloid A1</td>
<td>transporter</td>
<td>-3.99</td>
<td>AHR2h_700</td>
<td>Yes</td>
</tr>
<tr>
<td>Slc6a6</td>
<td>solute carrier family 6 (neurotransmitter transporter, taurine), member 6</td>
<td>transporter</td>
<td>1.79</td>
<td>AHR2h_1183</td>
<td>No</td>
</tr>
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<td>Thrsp</td>
<td>thyroid hormone responsive</td>
<td>other</td>
<td>-1.70</td>
<td>AHR2h_598</td>
<td>Yes</td>
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<tr>
<td>Gene</td>
<td>Description</td>
<td>Interactome</td>
<td>Fold Change</td>
<td>AHR-bound at 2h (1% FDR)</td>
<td>Differentially regulated genes at 4h (</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tiparp</td>
<td>TCDD-inducible poly(ADP-ribose) polymerase</td>
<td>other</td>
<td>3.61</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 10, AHR&lt;sub&gt;2h&lt;/sub&gt; 821</td>
<td>Yes</td>
</tr>
<tr>
<td>Tnfaip2</td>
<td>tumor necrosis factor, alpha-induced protein 2</td>
<td>other</td>
<td>2.90</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 261</td>
<td>Yes</td>
</tr>
<tr>
<td>Tnfaip8l1</td>
<td>tumor necrosis factor, alpha-induced protein 8-like 1</td>
<td>other</td>
<td>2.46</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 732</td>
<td>Yes</td>
</tr>
<tr>
<td>Tnfaip8l3</td>
<td>tumor necrosis factor, alpha-induced protein 8-like 3</td>
<td>other</td>
<td>3.03</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 340</td>
<td>Yes</td>
</tr>
<tr>
<td>Tnfrsf19</td>
<td>tumor necrosis factor receptor superfamily, member 19</td>
<td>transmembrane receptor</td>
<td>1.97</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 191, AHR&lt;sub&gt;2h&lt;/sub&gt; 611</td>
<td>Yes</td>
</tr>
<tr>
<td>Zadh2</td>
<td>zinc binding alcohol dehydrogenase domain containing 2</td>
<td>enzyme</td>
<td>1.61</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 29</td>
<td>Yes</td>
</tr>
<tr>
<td>Zfp395</td>
<td>Zinc finger protein 395</td>
<td>other</td>
<td>1.55</td>
<td>AHR&lt;sub&gt;2h&lt;/sub&gt; 114, AHR&lt;sub&gt;2h&lt;/sub&gt; 176</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<sup>1</sup> AHR-bound at 2h (1% FDR), differentially regulated genes at 4h (|fold change ≥ 1.5 and p ≤ 0.01)

<sup>2</sup> As determined by Genomatix MatInspector at a matrix similarity cutoff of 0.75

<em>N.B.</em> Cyp1a1 AHRE is not tiled on the array, and hence not included in the list.
Figure 24 Overlap between genomic AHR binding sites and TCDD-responsive gene expression in mouse hepatic tissue.

(A) Genes corresponding to AHR-bound regions after 2 h of TCDD treatment were overlapped with TCDD-responsive genes identified in our expression microarray array at 4 h. Venn diagram showed 39 TCDD-responsive genes directly regulated by AHR. (B) The expression levels of 18 genes corresponding to the 19 confirmed AHR-bound regions shown in Figure 22 were determined using qPCR. Results represent the mean values from four animals. Statistical significance (p < 0.05 compared to corn oil) is determined using the student’s t-test and is indicated with an asterisk.

Biological and toxicological pathways associated with TCDD exposure and AHR activation

We next identified the biological and toxicological pathways associated with the 39 TCDD-regulated and AHR bound genes using IPA analysis. These studies revealed that TCDD modulated multiple biological and physiological processes through AHR activation. Functions were divided into 1) adaptive response, 2) cellular and molecular response, 3) development and growth, and 4) diseases and disorders to illustrate the adaptive, toxic, and
developmental pathways activated by AHR. The top five functions in each category are shown in Table 5. We observed significantly enriched functions \( (p < 0.01) \) reflective of the adaptive/metabolic pathway including lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism, drug metabolism and nucleic acid metabolism. Functions related to growth and development in the endocrine system, respiratory system, connective tissue, and embryonic development were also significantly enriched. On the cellular level, AHR activation by TCDD affected functions involved in the regulation of cell death and cell cycle, whereas on the whole organism level, activation of AHR by TCDD was implicated in cancer, endocrine system disorders, genetic disorder, reproductive system disease, and infection mechanism.
Table 5. Functional Annotation Analysis on TCDD-responsive AHR-regulated genes using Ingenuity Pathway Analysis.

<table>
<thead>
<tr>
<th>Biological Functions</th>
<th>p-values</th>
<th># of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adaptive Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid Metabolism</td>
<td>$3.18 \times 10^{-6}$</td>
<td>10</td>
</tr>
<tr>
<td>Small Molecule Biochemistry</td>
<td>$3.18 \times 10^{-6}$</td>
<td>13</td>
</tr>
<tr>
<td>Vitamin and Mineral Metabolism</td>
<td>$9.3 \times 10^{-6}$</td>
<td>4</td>
</tr>
<tr>
<td>Drug Metabolism</td>
<td>$9.54 \times 10^{-6}$</td>
<td>5</td>
</tr>
<tr>
<td>Nucleic Acid Metabolism</td>
<td>$8.86 \times 10^{-6}$</td>
<td>2</td>
</tr>
<tr>
<td><strong>Cellular and Molecular Response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Death</td>
<td>$2.83 \times 10^{-6}$</td>
<td>15</td>
</tr>
<tr>
<td>Gene Expression</td>
<td>$3.17 \times 10^{-5}$</td>
<td>10</td>
</tr>
<tr>
<td>Protein Synthesis</td>
<td>$6.65 \times 10^{-5}$</td>
<td>2</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>$8.38 \times 10^{-5}$</td>
<td>7</td>
</tr>
<tr>
<td>Cellular Function and Maintenance</td>
<td>$4.33 \times 10^{-4}$</td>
<td>8</td>
</tr>
<tr>
<td><strong>Growth and Development</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocrine System Development and Function</td>
<td>$3.18 \times 10^{-6}$</td>
<td>5</td>
</tr>
<tr>
<td>Respiratory System Development and Function</td>
<td>$3.17 \times 10^{-6}$</td>
<td>3</td>
</tr>
<tr>
<td>Tissue Morphology</td>
<td>$3.17 \times 10^{-5}$</td>
<td>6</td>
</tr>
<tr>
<td>Connective Tissue Development and Function</td>
<td>$5.33 \times 10^{-4}$</td>
<td>8</td>
</tr>
<tr>
<td>Embryonic Development</td>
<td>$7.44 \times 10^{-4}$</td>
<td>7</td>
</tr>
</tbody>
</table>
**Histone modifications and coactivator recruitment at AHR target genes**

To determine whether changes in post-translational histone modifications at AHR-bound regions correlated with TCDD-dependent changes in target gene expression, we examined acetylation of lysine 9 (H3K9Ac) and dimethylation of lysine 4 of histone H3 (H3K4Me2) in hepatic tissue after 4 h of TCDD treatment, which matched the 4 h TCDD treatment used for the gene expression studies (Figure 24). H3K9Ac is associated with increases, whereas reduction in the level of H3K4Me2 is indicative of transcriptional activation (Kouzarides, 2007). These two histone modifications have been associated with activation of AHR-mediated transcription (Schnekenburger et al., 2007). Three positively-regulated and three negatively-regulated genes were chosen to assess how changes in H3K9Ac and H3K4Me2 correlated with gene expression changes in vivo. The three positively-regulated genes chosen were Cyp1b1 (AHR2h_14), Nfe2l2 (AHR2h_4), and Nqo1 (AHR2h_579), whereas the three non-responsive genes included Gabarap (AHR2h_1), Erbb3 (AHR2h_311) and Ppara (AHR2h_187). Gabarap was of particular interest due to the high basal binding of AHR to its upstream regulatory region and that its expression was not TCDD-responsive. AHR occupancy at the six regions after 4h TCDD treatment was also confirmed (data not shown). TCDD treatment resulted in increased H3K9Ac levels (expressed as a ratio of total H3 content) at Cyp1b1, Nfe2l2 and Nqo1 (Figure 25). Loss of H3K4Me2 was observed at Nfe2l2 and Nqo1, but not at Cyp1b1. In the case of Cyp1b1 the loss in histone H3 offset the reduction in H3K4Me2...
resulting in no changes in H3K4Me2:H3 ratio. The three TCDD non-responsive genes exhibited no distinct pattern in H3K4Me2 changes with significant reductions at \textit{Ppara} and \textit{Erbb3}, but no changes at \textit{Gabarap} (Figure 25). However, none of the TCDD-non-responsive genes showed an increase in H3K9Ac, which is in agreement with the lack of changes in target gene expression.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure25.png}
\caption{AHR-mediated and TCDD-induced gene expression was associated with an increase in H3K9Ac but not H3K4Me2. Acetylation at lysine 9 on Histone H3 (H3K9Ac) and dimethylation at lysine 4 on Histone H3 (H3K4Me2) were determined after 4 h of TCDD treatment. Cyp1b1, Nfe2l2 and Nqo1 were chosen to represent TCDD-responsive genes differentially regulated by AHR whereas Gabarap, Ppara, and Erbb3 were chosen to represent AHR-bound genes whose expression was not affected by TCDD. A significant increase in H3K9Ac was detected at the Cyp1b1, Nfe2l2, and Nqo1 AHR binding sites, but not at the Gabarap, Ppara, and Erbb3 AHR binding sites. There was no clear H3K4Me2 pattern associated with gene regulation at 4 h in vivo. Histone modifications were reported as a fraction of total Histone H3. Results represent the mean values from four animals. Statistical significance (p < 0.05 compared to corn oil) is determined using the student’s t-test and is indicated with an asterisk.}
\end{figure}
Discussion

In this study, we identified genomic regions bound by AHR in livers of female C57BL/6 mice after treatment with TCDD for 2h or 24h and analysis of promoter focused mouse tiling arrays. We observed a good overlap in AHR-bound regions between the AHR$_{2h}$ and AHR$_{24h}$ data sets with many of the well-known AHR target genes being detected in the assay (i.e. Cyp1b1, Nqo1 Nfe2l2 and TipARP). Although the role that each of the identified genes play in AHR-mediated signaling remains to be fully investigated, the AHR-bound regions add to the list of target genes and gene networks that contribute to AHR-mediated signaling and TCDD-induced toxicity. Previous AHR ChIP-chip studies using promoter-focused microarrays were done using immortalized mouse (Sartor et al., 2009) or human cell lines (Ahmed et al., 2009; Pansoy et al., 2010), whereas here we describe hepatic AHR-bound genomic regions in vivo. As expected, we found an over-representation of AHREs in the AHR$_{2h}$, AHR$_{24h}$ and AHR$_{overlap}$ regions, supporting a strong role for the AHRE and DNA-binding in AHR signaling (Bunger et al., 2008). However, since AHREs were not found in all regions, our study suggests the AHR is more promiscuous in its ability to bind chromatin (Gouedard et al., 2004; Pansoy et al., 2010). Furthermore, since not all AHR-bound regions resulted in changes in the expression for the corresponding genes, our findings show that AHR recruitment alone is not sufficient for gene regulation. The AHR-bound regions described here can be used to refine or improve the predictability of AHR binding sites in vivo.

AHR bound to some genomic regions in the absence of TCDD treatment. This was evident at Gabarap (AHR$_{2h,1}$) and Nfe2l2 (AHR$_{2h,4}$), with TCDD treatment increasing AHR occupancy at Nfe2l2, but not at Gabarap. AHR binding to chromatin in the absence of exogenous ligands may be the result of gene-specific activation by endogenous AHR ligands (Denison and Nagy, 2003), nuclear/cytoplasmic shuttling of AHR, or due to ligand-independent activation by AHR phosphorylation (Pongratz et al., 1991). However, for the majority of the regions that we analyzed AHR occupancy was dependent on TCDD treatment with no difference in AHR occupancy compared to IgG controls. This suggests that AHR occupancy in the absence of exogenous ligand treatment is likely to be a minor component of AHR signaling in hepatic tissue.
Using transcription factor binding site analysis we identified a number of transcription factor binding motifs within 10 to 50 bp of putative AHREs. Several of the identified binding motifs serve as response elements for transcription factors known to interact with AHR including E2F and Sp1. Previous studies have shown that AHR is recruited to E2F-dependent promoters and displaces the coactivator p300 to repress transcription (Marlowe et al., 2004), whereas Sp1 enhances AHR-mediated expression of the human CYP1B1 (Tsuchiya et al., 2003). The over-representation of FKHD binding motif is intriguing given the chromatin remodeling and pioneering factor role of Forkhead proteins in nuclear receptor signaling (Carroll et al., 2005; Lupien et al., 2009).

Time course ChIP studies revealed distinct AHR-recruitment profiles to Cyp1a1 in vitro and in vivo. In Hepa1c1c7 cells, maximal AHR recruitment occurs after 0.5h of TCDD treatment and is reduced after 24h of exposure, whereas high levels of AHR occupancy at many AHR responsive genes were confirmed at 2h and 24h in vivo. Comparison of our AHR2h ChIP-chip results with those reported by Sartor et al. (2009) using Hepa1c1c7 cells reveal that 62 of the reported 747 AHR\textsubscript{TCDD} bound genes were present in our data set (Sartor et al., 2009). Moreover, 7 of 11 AHR-bound regions were confirmed in Hepa1c1c7 treated with TCDD for 30 min, while only 4 of 11 of them were confirmed after 24h treatment. These findings are consistent with the reported differences in AHR regulated genes in vivo and in vitro (Dere et al., 2006) and certainly reflect pharmacodynamic and pharmacokinetic differences between immortalized cell lines and whole animal models. However, epigenetic differences such as DNA methylation and/or loss of additional transcription factors may also contribute to the reduced or lack of AHR recruitment to select genomic regions.

Our gene expression microarray experiments identified 133 unique genes changes in response to TCDD. Approximately 30% of the TCDD-regulated genes at 4h also exhibited AHR binding. However, it is possible that induction or repression of genes bound by AHR require additional signals, such as other transcription factors or that the mRNAs exhibit different temporal expression patterns that were not detected at the time point examined. Nonetheless, our findings are in agreement with the percentage of overlap in AHR-bound and TCDD-regulated genes in a genome-wide analysis for AHR-binding sites in ovariectomized female mice (Dere et al., 2011). Ingenuity Pathway Analysis revealed that in
addition to xenobiotic metabolism, AHR directly regulated a number of genes involved in cell death and cell cycle regulation, which is in agreement with previous reports (Boverhof et al., 2006; Moffat et al., 2010). Our results support previous studies demonstrating that AHR occupancy does not necessarily lead to changes in gene expression (Ahmed et al., 2009; Sartor et al., 2009). Since differential expression levels of coregulatory proteins and epigenetic changes are important molecular events that determine the transcriptional outcome at AHR target genes (Hankinson, 2005), liver specific expression of coregulatory proteins or chromatin modifiers may partially explain the lack of transcriptional outcome despite recruitment of AHR. Determining AHR-binding profiles in different mouse tissues will be important to determine if a common AHR-binding signature exits and whether additional tissue-specific coregulators determine transcriptional outcomes rather than recruitment of AHR to specific target genes.

As expected, 34 of the 39 TCDD responsive genes bound by AHR contain an AHRE core, confirming the important role of the AHRE in AHR signaling. However, these data also suggest that AHR can mediate gene expression responses through a non-AHRE-mediated mechanism. AHR can interact with non-AHRE genomic regions indirectly by tethering to other transcription factors, such as E2F, Sp1 and retinoblastoma (pRb) (Marlowe et al., 2004; Tsuchiya et al., 2003) (Ge and Elferink, 1998). Although only 53% of AHR-bound regions contained a core AHRE in the AHR2h regions, when the transcription factor binding site analysis was expanded to also include E2F-Myc and Sp1 binding motifs, these three motifs were found at least once in 86% of the AHR2h regions. This suggests that tethering through transcription factors such as E2F-Myc and Sp1 plays a significant role in the gene-specific recruitment of AHR. Alternatively, it is possible that AHRE-containing sequences not present on the promoter-focused arrays but bound by AHR are involved in the AHR-dependent transcription by bridging multiple genomic enhancer modules that lack core AHREs. Recent AHR ChIP-chip studies using whole genome tiling arrays to map AHR-bound regions in TCDD treated murine lymphoma CH12.LX B-cells that identified 1,893 regions but only 55% of the regions mapped to within 10 kb of transcription start site of known genes (De Abrew et al.). Similarly, our own whole genome tiling array analysis of AHR binding sites in livers of TCDD-treated ovariectomized immature C57BL/6 revealed that only 32% of all AHR binding sites were situated 10 kb upstream from annotated genes.
(Dere et al., 2011). These data show that the distribution of AHR binding sites extend beyond the coverage area of our promoter-focused array and that distal AHR bound regions might also contribute to AHR-mediated transcription. ChIA-PET (chromatin interaction analysis using paired-end tag sequencing) which combines 3C chromosome capture assays with high-throughput sequencing reveals that estrogen receptor regulates its target genes by interacting with multiple genomic sequences via a “looping” mechanism (Li et al., 2010). Such experiments will be important to determine if AHR were to use similar long range transcriptional mechanism to regulate target gene expression.

In summary, we report the identification of hepatic TCDD-responsive AHR-bound genes in vivo. The AHRE is the predominant transcription factor motif enriched in our data, supporting its central role in AHR signaling. The lack of a core AHRE in all of the AHR-bound regions, however, suggests that AHR exhibits a wider DNA-binding potential and/or binds to regulatory regions through tethering to other DNA-bound transcription factors. The data generated from our analysis identify AHR-bound regions in vivo, identify direct AHR target genes and represent a useful data set to refine AHR/DNA-binding predictions.
Significance of Study

Our study was one of the first to identify genome-wide, promoter-focused AHR binding sites in the mouse hepatic tissue and to compare the recruitment profiles of AHR in vivo and in vitro. Genome-wide binding site analysis coupled to gene expression profile analysis in animals exposed to TCDD allowed us to identify the primary effect of AHR activation in mouse liver in vivo. Our pathway analysis of AHR-bound and TCDD responsive genes confirmed the well-established role that AHR plays in the regulation of Phase I and II enzymes in the liver. However, in addition to the prototypical AHR target genes such as CYP1A1 and CYP1B1, AHR interacted with many previously uncharacterized AHR binding sites in the mouse genome, which might play a role in various biological processes including immune response and endocrine system development. This observation is in agreement with the fact that AHR has recently been proposed to be a potential therapeutic target for endocrine-related and/or immune-related diseases. Given our laboratory's interest in breast cancer and to address potential differences in AHR signalling between liver and breast tissues, we conducted similar genome-wide analysis of AHR binding sites in human MCF-7 breast cancer cells, which is presented in Chapter 4.

From a molecular mechanistic point of view, we elaborated on the genomic action of AHR. Interestingly, although the AHRE was significantly enriched in our AHR-bound regions, not all AHR-bound regions contained an AHRE, suggesting a strong, but not an absolute relationship between AHR-DNA interaction and the presence of an AHRE. AHR-DNA interaction in the absence of an AHRE might be mediated by interaction with other DNA-bound transcription factors, DNA looping, or other yet-to-be characterized mechanisms. Since our binding site analysis was limited to regions proximal to annotated transcription start site, we collaborated with Timothy Zacharewski who conducted genome-wide tiling analysis of AHR binding sites in mouse hepatic tissues. That study yielded similar results.
Chapter 4: High Resolution Mapping of Aryl Hydrocarbon Receptor (AHR) and AHR Nuclear Translocator Binding Sites Using ChIP-Seq

Raymond Lo and Jason Matthews

Based on Toxicological Sciences, in press

All experiments and analyses were performed by Raymond Lo. The final manuscript was written by Raymond Lo and Jason Matthews.

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Abstract

The aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) activated complex regulates genes in response to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). AHR has also emerged as a potential therapeutic target for the treatment of human diseases and different cancers, including breast cancer. To better understand AHR and ARNT signaling in breast cancer cells, we used chromatin immunoprecipitation linked to high throughput sequencing to identify AHR- and ARNT-binding sites across the genome in TCDD treated MCF-7 cells. We identified 2,594 AHR-bound, 1,352 ARNT-bound and 882 high confidence AHR/ARNT co-bound regions. No significant differences in the genomic distribution of AHR and ARNT were observed. Approximately 60% of the co-bound regions contained at least one core AHRE, 5’-GCGTG-3’. AHR/ARNT peak density was the highest within 1 kb of transcription start sites (TSS); however, a number of AHR/ARNT co-bound regions were located as far as 100 kb from TSS. *De novo* motif discovery identified a symmetrical variation of the AHRE (5’-GTGCGTG-3’), as well as FOXA1 and SP1 binding motifs. Microarray analysis identified 104 TCDD responsive genes where 98 genes were up-regulated by TCDD. Of the 104 regulated genes, 69 (66.3%) were associated with an AHR- or ARNT-bound region within 100 kb of their TSS. Overall our study identified AHR/ARNT co-bound regions across the genome, revealed the importance but not absolute requirement for an AHRE in AHR/ARNT interactions with DNA, and identified a modified AHRE motif, thereby increasing our understanding of AHR/ARNT signaling pathway.
Introduction

The aryl hydrocarbon receptor (AHR) belongs to the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) family of transcription factors (McIntosh et al. 2010). Other members of this family include hypoxia inducible factor 1 alpha (HIF-1α), AHR nuclear translocator (ARNT; HIF-1β), single-minded (SIM) and the AHR repressor (AHRR). The AHR mediates the toxic effects of environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). AHR binds and is activated by numerous structurally diverse natural and synthetic chemicals (Denison and Nagy 2003). The AHR regulates an array of physiological responses including xenobiotic metabolism, vasculature development, immunosuppression, T-cell differentiation, reproduction, and cell cycle progression (Bock and Kohle 2006). AHR has also recently emerged as a potential therapeutic target for immune disorders (Pot 2012) and for the treatment of different cancers, including breast cancer (Safe and Wormke 2003).

In its non-liganded state, the AHR resides in the cytoplasm bound to a chaperone protein complex that includes heat shock protein 90, AHR interacting protein, and p23 (Hankinson 1995). Once activated by ligand, AHR translocates into the nucleus where it associates with its obligatory heterodimerization partner ARNT. The AHR/ARNT heterodimer binds to its cognate DNA sequence motif, referred to as an aryl hydrocarbon response element (AHRE; 5′-TNGCGTG-3′) with a minimum core sequence of 5′-GCGTG-3′ required for AHR/ARNT binding (Swanson et al. 1995). Once bound to chromatin, the activated AHR/ARNT heterodimer induces the recruitment of coregulator proteins resulting in changes in target gene expression, including cytochrome P450 1A1 (CYP1A1), CYP1B1, nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; NRF2) and AHR repressor (AHRR) (Baba et al. 2001; Miao et al. 2005; Whitlock 1999; Zhang et al. 1998). Recent chromatin immunoprecipitation assays coupled with high density microarrays (ChIP-chip) have identified novel genomic sequences bound by AHR, providing new insight into the binding preferences across the genome (Ahmed et al. 2009; De Abrew et al. 2010; Dere et al. 2011; Lo et al. 2011; Pansoy et al. 2010; Sartor et al. 2009). However, compared to newer technologies such as chromatin immunoprecipitation coupled with next-generation high-throughput sequencing (ChIP-Seq), tiled array based approaches have a lower signal-to-noise
ratio, exhibit hybridization biases and are limited to the sequences present on the array (Wold and Myers 2008).

ARNT is a class II bHLH PAS transcription factor and the general heterodimerization partner for many bHLH proteins including AHR, hypoxia-inducible factor 1 and 2 (HIF-1α and 2α) and single-minded protein 1 and 2 (SIM1 and 2) (McIntosh et al. 2010). Conditional knockout of ARNT prevented gene regulation by AHR and HIF-1α, confirming a critical role of ARNT in AHR and HIF-1α signaling (Tomita et al. 2000). Given the requirement for ARNT in the transactivation of the AHR and HIF-1α, identifying genomic binding sites recognized by both partners of the heterodimerization complex would provide a more accurate definition of the DNA recognition sequences after receptor activation. For example, Schödel et al. recently reported extensive overlap between HIF-1α and ARNT (referred to as HIF-1β in their study) in 356 distinct genomic regions using ChIP-Seq (Schödel et al. 2011). The high confidence HIF-1α and ARNT co-bound regions reported in that study provided new insight into how the HIF-1α/ARNT complex interactions with chromatin and regulates target gene expression across the genome. Despite numerous studies describing the genomic binding patterns of AHR, there have been no reports investigating the overlapping binding profiles of ligand activated AHR and ARNT (Ahmed et al. 2009; De Abrew et al. 2010; Dere et al. 2011; Lo et al. 2011; Pansoy et al. 2010; Sartor et al. 2009). The identification of high confidence AHR/ARNT co-bound genomic sequences would improve our understanding of AHR transactivation, AHR interactions with chromatin and provide a robust data set that can be used to re-evaluate the AHR/ARNT DNA recognition sequence. To this end we performed ChIP-Seq and mapped AHR and ARNT co-bound genomic regions in TCDD-treated MCF-7 human breast cancer cells.
Materials and Methods

Chemicals and Antibodies

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Oakville, ON), whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Wellington Laboratories Inc. (Guelph, ON). Antibodies against AHR (H-211, sc-5579 Lot # K1010), and ARNT (H-172, sc-5580, lot #G3010) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Validation of ARNT-bound regions was performed using an anti-ARNT antibody (NB-100-110) from Novus Biologicals Inc. (Oakville, ON). All other reagents used were of the highest quality and scientific standards.

Cell Culture

MCF-7 cells were incubated at 37°C and 5% CO₂ and maintained in DMEM 1.0 g/L glucose (Wisent, St.-Bruno, QC) supplemented with 10% fetal bovine serum (FBS; Wisent, St.-Bruno, QC) and 1% penicillin and streptomycin (PEST; Wisent). Cells were plated in phenol red free DMEM 1.0 g/L glucose supplemented with 2.5% dextran-coated charcoal treated fetal calf serum (DCC-FCS) and 1% PEST for approximately 72 h before treatment.

Chromatin Immunoprecipitation with Next Generation Sequencing (ChIP-Seq)

MCF-7 cells were plated at a density of 3 million per 10 cm dishes. Conventional ChIP assays were conducted as described previously (Matthews et al. 2007). Immunoprecipitated DNA was eluted in a final volume of 50 μL and 20 μL was separated by electrophoresis using a 1.0% agarose gel. DNA fragments in the 200 - 400 bp range were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Maryland, USA), eluted in a final volume of 10 μL, and amplified using SeqPlex according to manufacturer’s protocol (Sigma Aldrich, St. Louis, MO). Library preparation and high-throughput sequencing were performed at BGI (Shenzhen, China). Briefly, isolated DNA was end-repaired to create a 3’-dA overhang. Adapters were ligated to the end of DNA fragments. DNA fragments between 100 - 500 bp were selected after PCR amplification and sequenced using the HiSeq2000 (Illumina Inc., San Diego, CA) for a final sequencing depth of 20 million reads per sample, 50 bp per read. Sequences were aligned to the human genome version 19 (Hg19) using Short Oligo Analysis Package 2.21 (BGI, Shenzhen, China) (Li et al. 2009). Enriched peaks were
normalized and analyzed using the two samples peak calling function in CisGenome (Ji et al. 2008) by comparing AHR and ARNT against duplicates of non-immunoreactive IgG. Regions with a minimum length of 100 bp were extended by 150 bp and regions less than 50 bp apart were merged. Confirmation of AHR-bound and ARNT-bound regions was performed on extracts from DMSO or TCDD-treated MCF-7 cells that were immunoprecipitated with normal IgG, anti-AHR antibody (H-211, sc-5579 Lot # K1010) from Santa Cruz Biotechnology and anti-ARNT antibody (NB100-110) from Novus Biologicals.

**Transcription Factor Binding Site Analysis**

The presence of an AHRE was determined using a family of matrices for the AHR-ARNT heterodimers and AHR-related factors available at MatInspector (www.genomatix.de). Sequences with a matrix similarity score of at least 0.8 were considered AHRE containing regions. Over-represented transcription factor binding site analysis was determined using the default parameter in RegionMiner (www.genomatix.de) based on the number of matches in immunoprecipitated DNA compared to the number of expected matches in genomic background. Module families and matrices were considered significant at $z$-score > 3. To identify transcription factors that might work in concert with AHR to modulate AHR transcription, we also determined the over-representation of transcription factor binding motifs located within 10 to 50 bp from putative AHREs in the AHR/ARNT-bound regions. Information on the definitions of the family matrices discussed herein is available on the Genomatix web page (www.genomatix.de). *De novo* motif discovery was conducted using the Gibbs motif sampler in CisGenome using a mean motif length of 12 bp and a maximum length of 30 bp. Significantly over-represented motifs extracted from Gibbs motif sampler were compared to existing binding motifs from the JASPAR (Wasserman and Sandelin 2004) and TRANSFAC (Matys et al. 2003) libraries using STAMP (Mahony and Benos 2007).

**RNA extraction and Gene expression microarray analysis**

MCF-7 cells were plated at a density of 350,000 per well. Total RNA was extracted using the Aurum Total RNA Mini Kit according to the manufacturer's recommendations (Bio-Rad,
Toronto, ON). RNA was prepared using the Ambion WT kit and hybridized to the Affymetrix Human Exon 1.0 ST array at the Toronto Centre for Applied Genomics (Toronto, Canada). Differentially regulated genes were identified with the Partek Genomics Suite (Partek Inc, St Louis, MO) using a false detection rate of 5% (FDR5) and an absolute fold change of greater than 1.20 between TCDD and DMSO treated samples.

**Functional analysis**

Canonical and function pathways significantly enriched by TCDD through AHR/ARNT binding were determined using Fisher’s exact test with the Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood CA) software.

**Statistical Analyses**

Statistical analysis was performed using analysis of variance with Bonferroni post test at \( p < 0.05 \). The significance values of biological functions/diseases identified in the IPA studies were calculated using the right-tailed Fisher’s exact test.
Results

Identification of AHR/ARNT binding sites

To identify high-resolution genomic AHR/ARNT binding sites, we performed ChIP-Seq on chromatin isolated from MCF-7 breast cancer cells exposed to 10 nM TCDD for 45 min. The experimental conditions were selected to best represent initial and maximal AHR/ARNT binding events that occurred prior to transcriptional changes in response to TCDD exposure (Pansoy et al. 2010). As expected, TCDD exposure resulted in the enrichment of multiple peaks in the previously characterized AHRE clusters upstream of the bidirectional promoter for the AHR target genes, *CYP1A1* and *CYP1A2* (Figure 26). We identified 2,594 AHR-bound region at a false detection rate ~1% (FDR1) and 1,352 ARNT-bound regions at FDR5. The FDRs were chosen to yield two datasets of similar size for comparison, while at the same time adjusting for differences in antibody specificity and affinity. AHR- and ARNT-bound regions were referred to as AHR_{number} and ARNT_{number}, respectively, where the number indicates the relative ranking within the analysis. The average peak lengths were 638 bp and 560 bp for AHR- and ARNT-bound regions, respectively. Locations of AHR- and ARNT-bound regions were divided into eight categories (intergenic, intragenic, exon, intron, coding DNA sequence [CDS], TSSup1k [1 kb upstream from TSS], TSSup10k, TSSup100k) based on available RefSeq annotation (Table 6). There were no significant differences (Fisher’s test, \( p > 0.05 \)) between the genomic distribution of AHR and ARNT datasets. Peak density (i.e. number of peaks/bp) was highest immediately adjacent to TSS (Figure 27 C and 27D) and promoter regions (500 bp upstream/100 bp downstream as defined by www.genomatix.de) were enriched by 3.6-fold in the AHR dataset and by 3.0-fold in the ARNT dataset. In terms of absolute numbers, AHR and ARNT binding sites were distributed such that ~2% of the binding sites were immediately upstream (1 kb), ~10% within 10 kb upstream and ~50% within 100 kb upstream from TSS. These findings support the notion that similar to members of the nuclear receptor family of transcription factors, AHR can regulate target gene expression through distal *cis*-acting elements.
Figure 26 Distribution of AHR and ARNT bound regions around six AHR regulated genes - *CYP1A1*, *CYP1A2*, *CYP1B1*, *AHRR*, *IGF1R* and *NRF2*.

A) Six TCDD responsive genes from our microarray experiments were chosen to represent the distribution of AHR and ARNT bound regions around their target genes. In the top track, black blocks represent AHR/ARNT co-bound regions identified by ChIP-Seq and are labeled according to their rank numbers in the AHR dataset. Shown in the middle track are the coding regions and introns of USCS genes, which are represented by blocks and connecting lines, respectively. The 5' and 3' untranslated regions (UTR) are indicated by thinner blocks, whereas arrows indicate the direction of transcription. In the bottom track, vertical lines indicate putative AHREs identified by MatInspector (Genomatix) at a core similarity of 0.75. AHR/ARNT co-bound regions were distributed in both intragenic and intergenic regions across large genomic distances. B) Confirmation of AHR and ARNT bound regions. All regions shown in Figure 26A were confirmed (ANOVA, p < 0.05) for AHR and ARNT recruitment by qPCR. Recruitment levels were shown as fold increase over IgG negative controls from at least two independent experiments and were represented by the color intensity on the
heat map. While some regions displayed constitutive AHR/ARNT occupancy, TCDD significantly increased AHR/ARNT recruitment to all regions.

High confidence AHR/ARNT binding sites were determined by the overlap of AHR- and ARNT-bound regions. Region overlap was determined based on a 100% sequence similarity and between regions that overlapped with >50% of the width of the smallest region using the ViroBlast web server (Deng et al. 2007). This resulted in 882 (65% overlap) high confidence AHR/ARNT bound regions (Figure 27 B). AHR/ARNT co-bound regions were labeled based on their corresponding rankings in the AHR dataset. The average peak height for the AHR/ARNT overlap dataset was 153.2, which was significantly higher than those for AHR (93.4) and ARNT (75.5) (Table 6).

Table 6. Genomic distribution of AHR and ARNT binding sites

<table>
<thead>
<tr>
<th></th>
<th>Peak Height</th>
<th># of Peaks</th>
<th>Intergenic</th>
<th>Intragenic</th>
<th>Exon</th>
<th>Intron</th>
<th>CDS 1 kb</th>
<th>10 kb</th>
<th>100 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR bound</td>
<td>93.4</td>
<td>2594</td>
<td>1508</td>
<td>1086</td>
<td>41</td>
<td>1051</td>
<td>15</td>
<td>58</td>
<td>261</td>
</tr>
<tr>
<td>ARNT bound</td>
<td>75.5</td>
<td>1352</td>
<td>797</td>
<td>555</td>
<td>20</td>
<td>537</td>
<td>6</td>
<td>27</td>
<td>134</td>
</tr>
<tr>
<td>Overlap</td>
<td>153.2</td>
<td>882</td>
<td>501</td>
<td>381</td>
<td>9</td>
<td>373</td>
<td>3</td>
<td>15</td>
<td>85</td>
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A) AHR and ARNT bound regions were defined as either intragenic (exon, intron, and CDS) or intergenic (TSSup1kb, TSSup10kb, and TSSup100kb). There were no significant differences (Fisher’s exact test, p > 0.05) between the genomic distribution of AHR and that of ARNT. B) AHR and ARNT bound regions containing at least one AHRE were identified by Genomatix (www.genomatix.de). Of the 882 AHR/ARNT co-bound regions, approximately 60% contained at least one AHRE. Distribution of AHR (C) and ARNT (D) bound regions indicated a binding preference for regions proximal to TSS. Peak density was the highest immediately adjacent to TSS for both AHR and ARNT, although the analysis also identified many distal AHR and ARNT bound regions.

**Figure 27** Genomic distribution of AHR and ARNT bound regions and the percentage of regions containing an AHRE.

**AHR/ARNT binding site analysis**

To evaluate the importance of the AHRE in the recruitment of the AHR complex, we interrogated the AHR, ARNT, and co-bound datasets using a family of AHRE position weight matrices (PWMs) available in MatInspector (www.genomatix.de). Approximately
(50.5% and 47.9% of the AHR and ARNT datasets contained at least one AHRE, whereas the percentage improved to 60% in the AHR/ARNT overlap dataset (Figure 27 B). These results were in agreement with previous reports showing that the AHRE was the major determinant, but not an absolute prerequisite for AHR/ARNT recruitment (Ahmed et al. 2009; De Abrew et al. 2010; Dere et al. 2011; Lo et al. 2011; Pansoy et al. 2010; Sartor et al. 2009).

AHR has been reported to interact with an alternate response element termed AHRE-II (5'-CATGnnnnC[T/A]TG-3') (Boutros et al. 2004; Sogawa et al. 2004). Transcription factor binding sites analysis was performed to determine the number of AHR/ARNT bound regions that contained an AHRE-II site. We determined that 116/882 (13.1%) AHR/ARNT-bound regions contained at least one AHRE-II. Of the 353 AHR/ARNT bound regions that did not contain an AHRE core, 38 of them contained an AHRE-II site. However, only one TCDD-regulated gene contained an AHRE-II site without a core AHRE in its upstream regulatory region. These findings confirmed the importance of the AHRE in comparison to the AHRE-II in AHR/ARNT interactions with DNA.

De novo motif discovery of a symmetrical AHRE

To address the possibility of an alternate AHR/ARNT binding motif and to improve the existing AHRE binding motif which was based on in vitro AHR-DNA interaction and mutation analysis of the CYP1A1 AHRE (Shen and Whitlock 1992; Swanson et al. 1995), we did de novo motif discovery on the top 500 regions in AHR-bound, ARNT-bound, and overlap datasets using Gibbs motif sampler in CisGenome. The unsupervised analysis identified the previously reported core AHRE (5'-GCGTG-3') in all three datasets, but not the extended AHRE (5'-TnGCGTG-3') (Swanson et al. 1995) (Figure 28). PWM indicated a strong preference for a guanine and a thymine flanking the 5' end of the core AHRE, resulting in an extended and symmetrical motif with the sequence 5'-GTGCGTG-3' (highlighted in dotted line box in Figure 28 A). De novo motif discovery was repeated using a web-based application W-ChIPMotifs (Jin et al. 2009), which confirmed the symmetrical motif 5'-GTGCGTG-3' (data not shown). Our motif matrices from the AHR/ARNT overlap dataset were compared to the PWM library from JASPAR and TRANSFAC using STAMP (Figure 28 B). In addition to the symmetrical AHRE, Gibbs motif sampler identified
additional binding motifs that aligned to MA0277.1_AZF1, MA0148.1_FOXA1, and MA0079.2_SP1.

Figure 28 Discovery of novel binding motifs for AHR and ARNT bound regions.

De novo motif discovery was performed using Gibbs motif sampler in CisGenome (Ji et al. 2008). A) A symmetrical variation (highlighted in dotted box) of the AHRE was identified in all three datasets. Comparison to known STAMP and TRANSFAC motifs identified the AHRE as the most probable candidate binding motif. B) De novo discovery yielded three motifs in addition to the AHRE. STAMP identified those three motifs to be AZF1, FOXA1, and SP1.

Transcription factor binding site enrichment analysis

To identify transcription factor binding motifs that were over-represented in the AHR-, ARNT- and co-bound regions we did transcription factor binding site enrichment analysis using RegionMiner (www.genomatix.de; Table 7). As expected, RegionMiner detected a significant over-representation of AHR_ARNT binding sites in the AHR-bound, the ARNT-bound, and the overlap datasets. Other notable over-represented binding motifs included early growth response 1 (EGR1), activator protein family (AP1 and AP2), SP1, estrogen
receptor (ER), and hypoxia-inducible factor (HIF). The antioxidant response element recognized by NRF2 was also over-represented in all three datasets, which was in agreement with noted interplay between AHR-NRF2 signaling (Yeager et al. 2009). To identify other transcription factors that might work in concert with AHR to modulate AHR function we searched the AHR/ARNT regions for enriched transcription factor motifs located within 10 to 50 bp of AHREs using RegionMiner (Genomatix). This analysis found enrichment of AHREs in close proximity to EGR, AP1, homologues of enhancer of split complex (HES1) as well as forkhead box (FKHD), suggesting possible physical interaction or co-regulation of target genes between the candidate transcription factors and AHR (Table 8). Interestingly, some module pairings displayed spatial preferences for interaction. For example, the Maf_AP1 related factors (AP1R) binding motif and the ERE were uniformly distributed around an AHRE, whereas the pairing of AHREs and FKHD binding motifs appeared to have a spatial preference of > 25 bp apart in our AHR/ARNT co-bound regions.

Table 7. Analysis of Enriched Transcription Binding Sites

<table>
<thead>
<tr>
<th>TF Families</th>
<th>TF Description</th>
<th>Number of Matches</th>
<th>Expected</th>
<th>Z-Score</th>
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<tbody>
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<td>MAF and AP1 related factors</td>
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<tr>
<td></td>
<td>(Antioxidant Response Element)</td>
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<td></td>
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<td>SP1F</td>
<td>GC-Box factors SP1/GC</td>
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### Top 10 Enriched Transcription Factor Binding Sites in AHR-Bound Regions

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### Top 10 Enriched Transcription Factor Binding Sites in ARNT-bound Regions

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Table 8. Analysis of Enriched Transcription Binding Sites within 10 – 50 bp from an AHRE

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<th>Modules with AHR</th>
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<th>Number of Matches</th>
<th>Expected</th>
<th>Z-Score</th>
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<td>AHR_P53F</td>
<td>p53 tumor suppressor</td>
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Confirmation of ChIP-Seq AHR/ARNT binding profiles using qPCR ChIP

AHR/ARNT co-bound regions and computationally determined AHREs were plotted using UCSC Genome Browser to investigate the relationship between the presence of an AHRE and AHR/ARNT recruitment to select AHR target genes. CYP1A1 and CYP1A2 are regulated by AHR/ARNT recruitment to their bidirectional promoter (Ueda et al. 2006) (Figure 27). In
addition to the well-characterized AHRE cluster located approximately 1 kb upstream from CYP1A1 TSS, we observed significant AHR/ARNT recruitment to a region 3’ of CYP1A2 that had been previously reported to be important for the AHR-dependent regulation of CYP1A2 (Okino et al. 2007). For CYP1B1, we confirmed AHR/ARNT recruitment to a region within 2 kb upstream of the TSS, which represents the proximal promoter (MacPherson and Matthews 2010) and upstream AHR-rich enhancer (Matthews et al. 2005). Our analysis also revealed AHR/ARNT binding to two novel regions approximately 15 kb upstream of the TSS (Figure 26). All AHR/ARNT co-bound regions associated with CYP1A1, CYP1A2 and CYP1B1 contained an AHRE core sequence with the exception of AHR1578.

Haarmann-Stemmann et al. identified a functional AHRE ~17 kb downstream of the AHRR TSS between exon 1 and exon 2 using reporter gene assays in HepG2 human hepatoma cells (Haarmann-Stemmann et al. 2007). We did not detect AHR/ARNT recruitment to the AHRE rich sequence between exon 1 and 2. We identified two AHR/ARNT co-bound regions in the intronic regions of AHRR, regions AHR708 and AHR2445 (Figure 26). Neither contained an AHRE core sequence. Interestingly, we also identified an AHRE cluster at approximately chr15: 390,000 - 395,000 between AHRR exon 5 and 6, but this was not associated with an AHR/ARNT bound region.

Three AHREs have been identified in the 2 kb surrounding the murine nrf2 TSS that might be involved in the AHR-dependent regulation of that gene (Miao et al. 2005). Comparison between the mouse and the human genome revealed at least five potential AHREs in the regulatory region of the human NRF2 (Miao et al. 2005), but to our knowledge, AHR or ARNT recruitment to these regions has not been experimentally confirmed. We identified and confirmed one AHR/ARNT-bound region (AHR638) upstream of the NRF2 start codon (Figure 26), which contained two AHREs.

Multiple AHR/ARNT co-bound regions were identified and confirmed in the intragenic regions of insulin-like growth factor 1 receptor (IGF1R). IGF1R is an estrogen-responsive gene that was positively regulated after 24 h of 0.1 nM TCDD exposure in vivo or in vitro (Tanaka et al. 2007). The AHR/ARNT co-bound regions were AHR1, AHR106, AHR230, AHR400, AHR711 and AHR786. Each of the regions contained at least one AHRE core sequence, with the exception of AHR786.
Independent ChIP assays confirmed TCDD-dependent recruitment of AHR and ARNT to all regions (Figure 27 B). We did not observe any false positives during our qPCR validation of binding sites, consistent with our high stringency cutoff of FDR1 and FDR5. In summary, AHR/ARNT bound regions were distributed in intergenic and intragenic regions across large genomic regions. Some AHR/ARNT bound regions were associated with a putative AHRE cluster, whereas others were not. These data supported a strong, but not an absolute relationship between AHR/ARNT binding and the presence of an AHRE cluster. They also revealed that AHR/ARNT bound to very small proportion of the computationally predicted AHRE across the genome.

Integration of AHR/ARNT binding events with changes in gene expression
We next performed gene expression microarray experiments in MCF-7 exposed to 10 nM TCDD for 6 h. The resulting dataset was then compared to genes associated with AHR- or ARNT-bound regions. We identified 104 unique differentially regulated genes at cutoff of $|\text{fold-change (FC)}| \geq 1.2$ and FDR5. The primary effect of TCDD on MCF-7 cells was gene activation since the vast majority (98 or 94.2%) of the regulated genes were significantly induced at 6 h. Of the 104 unique differentially regulated genes, 69 (66.3%) were associated with an AHR- or ARNT-bound region within 100 kb of their corresponding TSS (Figure 29 A). The distribution of AHR- or ARNT-bound regions within 100 kb of a TCDD responsive gene was centered at 2102 bp downstream from TSS (Figure 29 B). Peak enrichment was located in the proximal promoter area (within 10 kb), although a number of binding sites greater than 10 kb from the TSS of TCDD responsive genes were also present. A list of the 69 TCDD-responsive and AHR/ARNT-bound genes was provided in Table 9. Of the 69 genes, 59 (85.6%) contained at least one AHRE, representing TCDD-induced, AHR-mediated and AHRE-dependent gene regulation.

<p>| Table 9. Differentially Regulated Genes with an AHR or ARNT bound region within 100kb. |
|-----------------|--------------------|-----------------|-----------------|-----------------|
| Gene Symbol     | Genomic Coordinate | Genomic Location | Fold Change AHRE? |
|-----------------|--------------------|-----------------|-----------------|-----------------|
|                 |                    |                 |                 |                 |</p>
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Figure 29 A) Percentage of TCDD responsive genes that were associated with an AHR or ARNT bound region.

Approximately 66.3% (69/104) of TCDD responsive genes were associated with at least one AHR or ARNT bound region and 85.6% (59/69) of these regions contained at least one AHRE. B) Distribution of AHR/ARNT bound regions around TCDD responsive TSS. Peak enrichment is located at the proximal promoter, although there are many AHR/ARNT bound regions greater than 10 kb from TCDD responsive TSS as well.

Gene Function and Pathway Analysis

Function and pathway analysis was conducted on 69 TCDD-induced AHR or ARNT-bound genes to evaluate the direct impact of AHR activation at the cellular level. Pathway analysis of those 69 bound and regulated genes demonstrated significant changes in the AHR
signaling pathway, xenobiotics metabolism by CYP450, xenobiotic metabolism signaling, fatty acid metabolism and tryptophan metabolism. All of which correlated with the perturbation of cellular functions such as drug metabolism, small molecule biochemistry, nucleic acid metabolism and lipid metabolism (Table 10). This was consistent with our previous genome-wide studies on mouse hepatic tissue (Dere et al. 2011; Lo et al. 2011) and with the role of AHR as a regulator of xenobiotic metabolism and lipid homeostasis. AHR-ARNT was among the top predicted transcription factors along with AHR interacting protein (AIP), homeobox gene GSX2 and breast cancer 1, early onset (BRCA1).

Table 10. Enrichment of canonical pathways, functional pathways and predicted transcription factors for the 69 regulated and AHR/ARNT bound genes using IPA.

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Discussion

Our laboratory and others have described ligand activated genomic binding of AHR using promoter-focused and genomic-wide microarrays (Ahmed et al. 2009; De Abrew et al. 2010; Dere et al. 2011; Lo et al. 2011; Pansoy et al. 2010; Sartor et al. 2009); however, similar studies have not been described for ARNT. We report here the genome-wide analysis of AHR and ARNT binding sites in MCF-7 breast cancer cells. Our results reveal a high, albeit lower than expected overlap between AHR- and ARNT-bound regions. The AHRE sequence is over-represented in AHR/ARNT-bound regions, but only 60% contain at least one AHRE core sequence. These findings support previous studies demonstrating that AHR/ARNT heterodimer is promiscuous in its binding to DNA and not limited to AHREs (Ahmed et al. 2009; Dere et al. 2011; Lo et al. 2011; Pansoy et al. 2010). However, across the entire genome only a small proportion of these computationally predicted AHREs are bound by AHR/ARNT. Our analysis also identifies novel AHR-regulated bound regions in the regulatory regions of AHR target genes, including *AHRR* and *NRF2*. By integrating our ChIP-Seq data with gene expression microarray data we provide a list of high confidence AHR/ARNT-bound and TCDD responsive genes with significant changes occurring in genes involved in lipid metabolism and drug metabolism.

Schödel et al. recently reported genome-wide mapping of HIF-1α and ARNT (referred to as HIF-1β in their study), revealing a ~89% overlap (356/400) between HIF-1α and ARNT binding sites (Schödel et al. 2011) using a different ARNT antibody, NB100-110 from Novus Biologicals. The degree of overlap between AHR and ARNT in our study is lower (65%; 882/1,352) and might be due to differences in antibody affinity as the average peak height for the ARNT dataset is significantly lower than that for AHR. Differences in anti-ARNT antibody used might also account for this discrepancy. We are testing this hypothesis by repeating our ChIP-Seq studies with anti-ARNT antibody from Novus Biologicals (NB100-110). Comparison between the HIF-1α-ARNT binding sites reported by Schödel et al. (2011) in hypoxic cells and the AHR-ARNT bound regions in our study reveals minimal overlap, suggesting that the distribution of ARNT binding sites is heavily determined by its heterodimerization partner. These findings are also consistent with the fact that AHR and HIF-1α belong to two different molecular pathways with different sets of regulated genes and binding targets.
The lack of complete overlap between AHR-bound and ARNT-bound regions also suggests that both factors might interact independently with DNA. In support of this notion, AHR recruitment to a non-consensus AHRE in the murine plasminogen activator inhibitor-1 (PAI-1) has been reported to be independent of ARNT (Huang and Elferink 2012). PAI-1, however, is not associated with either AHR or ARNT-bound regions in our present study, consistent with our previous ChIP-chip data in T-47D human breast cancer cells (Ahmed et al. 2009). This suggests that PAI-1 might not be regulated by AHR in human breast cancer cells, which is not surprising given the well-documented species differences in AHR signaling (Flaveny et al. 2010). AHR- or ARNT-specific regions present in our dataset may be influenced by the statistical cut-offs as well as the ability of the different antibodies to recognize their targets under our assay conditions, leading to potential false negatives. For example, in many cases AHR-bound regions at FDR1 not bound by ARNT at FDR5 are present in ARNT-bound regions at a more relaxed FDR10. We are currently using zinc-finger nuclease strategies to generate AHR and ARNT knockout cell lines to use as models to identify TCDD-induced and specific AHR- or ARNT-bound sequences and target genes.

In agreement with previous reports, our transcription factor binding site enrichment analysis reveals the overrepresentation of AHRE, ERE, SP1 and ARE motifs in AHR/ARNT-bound regions (De Abrew et al. 2010; Dere et al. 2011; Lo et al. 2011; Sartor et al. 2009). Estrogen receptors and SP1 proteins have been previously reported to modulate AHR transactivation mechanisms (Ahmed et al. 2009; Kobayashi et al. 1996). NRF2, which regulates gene expression in response to oxidative stress through binding to AREs, is an AHR target gene but has also been shown to work in concert with AHR to regulate the expression of numerous genes, including *NADPH quinone oxidoreductase 1 (NQO1)* (Yeager et al. 2009). NRF2 knockout prevents AHR-dependent regulation of *NQO1* (Yeager et al. 2009), suggesting that *NQO1* is directly regulated by NRF2, with its TCDD-dependent regulation likely a secondary effect of AHR-mediated increase in NRF2 expression (Yeager et al. 2009). We also report at least one AHR-bound region for NRF2 target genes such as *NQO1*, *GSTP1* and *GSTM1-5*, supporting a close functional relationship between AHR and NRF2 in the regulation of shared target genes. Although not studied in a recent ChIP-Seq analysis of NRF2 sites in lymphoblastoid cells, transcription factor binding site enrichment analysis of the NRF2 sites reported by Chorley et al. reveals that the AHRE is significantly
over-represented (data not shown) (Chorley et al. 2012). Taken together, these findings further support the functional interactions between the two transcription factors.

FKHD motifs are enriched adjacent to an AHRE and a consensus FOXA1 binding motif is generated from our unsupervised de novo motif discovery using a set of AHR/ARNT-bound regions. FOXA1 is the major determinant for estrogen receptor binding where knockdown of FOXA1 can significantly reduce estrogen receptor recruitment at most loci (Hurtado et al. 2011). We recently reported that FOXA1 is required for AHR-dependent regulation of cyclin G2, but not for CYP1A1, supporting a gene selective role for FOXA1 in AHR transactivation (Ahmed et al. 2012). The impact of FOXA1 on AHR transactivation at the genome-wide level has not been evaluated.

Our de novo motif analysis identifies a core AHRE, but not the extended AHRE or the AHRE-II. This implies that although AHR interaction with the other two variations of the AHREs is possible under cell-type specific or gene specific context, the core AHRE remains one of the best predictor for AHR interactions with chromatin. The symmetrical AHRE (5’GTGCGTG’3) we describe is identical to the SIM/ARNT motif and similar to the second ranking AHR/ARNT motif reported by Swanson et al. (Swanson et al. 1995). The extended AHRE (5’TNGCGTG’3) is responsible for robust AHR/ARNT recruitment to the AHRE clusters for CYP1A1. However, the more flexible symmetrical AHRE might be a better predictor of AHR/ARNT binding sites throughout the genome.

We report 104 TCDD responsive genes using microarray gene expression analysis with ~95% of the regulated genes being up regulated with only 66.3% associated with an AHR- or ARNT-bound region. Furthermore, AHR/ARNT bound regions identified by ChIP-Seq vastly outnumber TCDD responsive genes in our expression microarray. These discrepancies between binding events and gene regulation may be due to temporal differences in mRNA expression (Boverhof et al. 2005), non-genomic effect of AHR activation, or the result of cell-type specific expression patterns through epigenetic modulation that are independent of AHR/ARNT occupancy. It also remains possible that the distal AHR/ARNT-bound regions might be involved in the regulation of non-protein coding transcripts, such as non-protein coding RNA, small nucleolar RNAs and microRNA, that would have been missed by our microarray studies. Future experiments using RNA-
sequencing will provide valuable information on the bridging of multiple DNA elements with both coding and non-coding transcriptome.

AHR/ARNT binds preferentially to proximal promoter regions as peak density was the highest in regions within 1 kb from transcription start sites, indicating that many AHR target genes are directly regulated by AHR binding to the proximal promoter. On the other hand, there are also many AHR/ARNT-co-bound regions located distal (~100 kb) from annotated transcription start sites (Table 6, Figure 27 C and D). Although more data are needed, our findings suggest that AHR-mediated gene regulation might involve long-range interaction of distal cis-regulatory regions in a mechanism similar to that of nuclear receptors. This is supported by previous studies showing that nuclear receptor binding sites are also distal to regulated genes both in vivo upon acute ligand stimulation (Boergesen et al. 2011; Hewitt et al. 2012) and in vitro (Carroll et al. 2006; Reddy et al. 2009). Gene regulation by remote cis-acting regions has been experimentally shown to occur through chromatin remodeling, DNA looping (Fullwood et al. 2009), or even inter- and intra-chromosomal interaction (Hu et al. 2008), making it challenging to correlate DNA interaction with downstream transcriptional outcomes.

Overall, our data are in agreement with previous studies supporting the important role of the AHRE in AHR transactivation. However the lack of a core AHRE in all of the AHR-bound regions, suggests that AHR exhibits more flexible DNA-binding potential. This is supported by the less stringent symmetrical AHRE motif predicted from our study and the identification of cooperative transcription factors that might mediate indirect AHR-DNA interaction. Our results provide a comprehensive genome-wide AHR/ARNT binding site analysis and a robust dataset that can be used to improve predictions for functional AHR/ARNT DNA interaction.
Significance of Study

Our data represented the most comprehensive genome-wide AHR/ARNT binding site analysis in human breast cancer to date. Our genome-wide analysis of AHR/ARNT binding sites demonstrated that the AHRE sequence is an important, but not an absolute requirement in AHR transactivation. Given that AHR is the key regulator of Phase I xenobiotic metabolism and a potential therapeutic target for the treatment of breast cancer, information from our study may be used to develop novel treatment strategies against breast cancer.

In addition to elucidating genome-wide AHR transactivation, we observed interplay between NRF2 and AHR signalling pathway. NRF2 is the key regulator of many Phase II detoxification enzymes. The interplay between NRF2 and AHR represents one potential mechanism to link the concerted regulation of Phase I and Phase II enzymes. NRF2-AHR crosstalk, therefore, might have important biological implications in modulating the production of genotoxic metabolites in the body. This observation led us to investigate potential interaction among NRF2, AHR, and ERα in the regulation of antioxidative enzymes such as NQO1 and HMOX1 described in Chapter 5.
Chapter 5: Aryl Hydrocarbon Receptor and Estrogen Receptor Modulate the Transcriptional Activity of Nuclear Factor Erythroid-2 Like 2

Raymond Lo and Jason Matthews

All experiments and analyses were performed by Raymond Lo. The accepted manuscript was written by Raymond Lo and Jason Matthews.

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MANUSCRIPT ACCEPTED FOR PUBLICATION IN TOXICOLOGY AND APPLIED PHARMACOLOGY
Abstract

Nuclear Factor Erythroid-2 like 2 (NRF2; NFE2L2) plays an important role in mediating cellular protection against reactive oxygen species. NRF2 signaling is positively modulated by the aryl hydrocarbon receptor (AHR) but inhibited by estrogen receptor alpha (ERα). In this study we investigated the crosstalk among NRF2, AHR, and ERα in MCF-7 breast cancer treated with the NRF2 activator sulforaphane (SFN), the dual AHR and ERα activator 3,3’-diindolylmethane (DIM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or 17β-Estradiol (E2). SFN-dependent increases in NADPH-dependent oxidoreductase 1 (NQO1) and heme oxygenase I (HMOX1) mRNA expression levels were significantly reduced after co-treatment with E2. E2-dependent repression of NQO1 and HMOX1 was associated with increased ERα but reduced p300 recruitment and reduced histone H3 acetylation at both genes. In contrast, DIM+SFN or TCDD+SFN induced NQO1 and HMOX1 mRNA expression to levels higher than SFN alone, which was prevented by RNAi-mediated knockdown of AHR. DIM+SFN but not TCDD+SFN also induced recruitment of ERα to NQO1 and HMOX1. However, the presence of AHR at NQO1 and HMOX1 restored p300 recruitment and histone H3 acetylation, thereby reversing the ERα-dependent repression on NRF2. Taken together, our study provides further evidence of functional interplay among NRF2, AHR, and ERα signaling pathways through altered p300 recruitment to NRF2-regulated target genes.
Introduction

Nuclear Factor-Erythroid 2 Related Factor 2 (NRF2; NFE2L2) plays an indispensable role in conferring cellular protection against electrophiles and oxidative stress by up-regulating the expression of Phase II detoxifying enzymes including *NADPH-dependent Quinone Oxidoreductase I and II* (*NQO1* and 2), *Heme Oxygenase I* (*HMOX1*), *Glutamate-cysteine Ligase Catalytic Subunit* (*GCLC*), and *gamma-Glutamyl-cysteine Synthetase* (*γ-GCS*) (Chanas et al., 2002; Kwak et al., 2001; Malhotra et al., 2010). NRF2, under normal circumstances, is negatively regulated by Kelch-like ECH-associated protein I (KEAP1) in the cytoplasm and is targeted for ubiquitination and proteolytic degradation (Zhang, 2006). Electrophiles and reactive oxygen species can irreversibly modify reactive cysteine residues in KEAP1 to inhibit KEAP1-NRF2 interactions. This initiates NRF2 stabilization, nuclear translocation and recruitment to genomic regions known as antioxidant response elements (ARE), leading to the regulation of anti-oxidative and cytoprotective genes (Zhang, 2006). Recent studies showed that the aryl hydrocarbon receptor (AHR) (Hayes et al., 2009; Yeager et al., 2009) and estrogen receptor alpha (ERα, *ESR1*; NR3A1) (Ansell et al., 2005) interacted with and modulated NRF2 activity. AHR activation positively, whereas ERα activation negatively affects NRF2 transactivation (Ansell et al., 2005; Montano and Katzenellenbogen, 1997; Yao et al.; Yeager et al., 2009). However, the combined effect of both AHR and ERα activation on NRF2 activity remains unclear.

ERα is one of two estrogen receptor subtypes found in the human genome along with ERβ (*ESR2*; NR3A2) (Nilsson and Gustafsson, 2000). ERα resides in the nucleus and belongs to the class I nuclear receptor superfamily. 17β-estradiol (E2) activates ERα and initiates a cascade of events, leading to ERα recruitment and subsequent gene regulation. ERα has been one of the strongest prognostic factors and is the primary therapeutic target for breast cancer (Pearce and Jordan, 2004). The AHR is a ligand-activated transcription factor and member of the basic helix–loop–helix (bHLH)-Per-ARNT-Sim (PAS) superfamily of transcriptional regulators. The AHR mediates the toxic effects of environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but is also activated by numerous structurally diverse natural and synthetic chemicals. When activated by ligand binding, the AHR translocates from the cytoplasm to the nucleus, heterodimerizes with the
aryl hydrocarbon receptor nuclear translocator (ARNT) and up-regulates a battery of Phase I metabolic enzymes (Hankinson, 1995) including cytochrome P450 (CYP) such as CYP1A1 and monooxigenases such as flavin monooxygenase 3 (FMO3). Reciprocal crosstalk between ERα and AHR has been well documented (Matthews and Gustafsson, 2006). Ligand activated AHR can influence the protein level of ERα and can influence the level of estrogen by up-regulating estrogen metabolizing cytochrome P450, resulting in reduced ERα activity (Lee et al., 2003). Furthermore, crosstalk can occur at specific genomic regions as studies have shown ectopic recruitment of ERα to AHR target genes and vice versa in breast cancer cells co-treated with dioxin and E2 (Ahmed et al., 2009).

Cruciferous vegetables such as broccoli sprouts contain high levels of glucosinolates, which can be broken down into isothiocyanates, such as sulforaphane, (a KEAP1 inhibitor and hence a NRF2 activator) and indoles such as indole-3-carbinol (I3C) (Hayes et al., 2008). In the gastrointestinal tract, I3C conjugates are hydrolyzed to many products including indolo[3,2-b]carbazole (ICZ) and 3,3′-diindolylmethane (DIM), which is an AHR agonist as well as an ER activator (Chen et al., 1998; Leong et al., 2004b; Vivar et al., 2010). Although a recent study reported synergistic increases in NRF2 transactivation following co-treatment with DIM and SFN (Saw et al., 2011), the role of AHR and ERα in mediating this synergism was not examined.

In this study we investigated the combined effect of SFN and DIM, and the resulting activation of AHR and ERα on NRF2-regulated target gene expression. We observed that ERα repressed NRF2 activity by interfering with p300 recruitment and subsequent histone acetylation at NRF2 target genes. Ligand activated AHR reversed the ERα-dependent repression of NRF2 action possibly by restoring histone acetylation of p300 recruitment to \textit{NQO1} and \textit{HMOX1}. Our data revealed a complex functional interplay among the NRF2, AHR, and ERα signaling pathways upon exposure to dietary compounds that can simultaneously activate all three receptor systems.
Materials and Methods

Chemicals and Antibodies

3,3′-diindolylmethane (DIM) was purchased from Enzo Life Sciences (Farmingdale, NY). Dimethyl sulfoxide (DMSO), 17β-estradiol (E2), D,L-sulforaphane (SFN) were purchased from Sigma-Aldrich (Oakville, ON). RNAi targeting ERα and AHR were purchased from Dharmacoa, Inc., Thermo Fisher Scientific (Lafayette, CO). Antibodies against ERα (HC20), NRF2 (C20), AHR (H211), and p300 (N15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against C-terminal Histone H3 (ab1791) was purchased from Abcam Inc. (Cambridge, MA) whereas antibody against Histone H3 Lysine 9 Acetylation (07-352) was purchased from Millipore (Billerica, MA). All other reagents used were of the highest quality and scientific standards.

Cell Culture

MCF-7 cells were maintained in DMEM with 1.0 g/L glucose (Wisent, St.-Bruno, QC) supplemented with 10% fetal bovine serum (FBS; Wisent, St.-Bruno, QC) and 1% penicillin and streptomycin (PEST; Wisent) and incubated at 37°C and 5% CO2. Prior to treatment, cells were steroid deprived for 72 h in phenol red free DMEM with 1.0 g/L glucose supplemented with 2.5% dextran-coated charcoal treated fetal calf serum (DCC-FCS) and 1% PEST.

Chromatin Immunoprecipitation (ChIP)

MCF-7 cells were plated at a density of 3 million per 10 cm dishes. ChIP assays were conducted as described previously (Pansoy et al., 2010). Sequences for ChIP primers were described by Ahmed et al. (Ahmed et al., 2009) for CYP1A1 and GREB1, by Reichard et al. (Reichard et al., 2007) for HMOX1, and by Dhakshinamoorthy et al. (Dhakshinamoorthy et al., 2005) for NQO1. NRF2 Region 1 was amplified with F 5’-TCGTCTCCCTTTCTTGTTGTCG-3’ and R 5’-ACTCAGAGCCTTTGGGGAGGA-3’ and NRF2 Region 2 with F 5’-GAAACGCAAGCGTTCCACTCTCTTGAGAAGG-3’ and R 5’-AACATGTTCCTCCCTTTGTGCAGGG-3’.
RNA extraction and cDNA synthesis

MCF-7 cells were plated at a density of 250,000 per well in a 6-well plate. Total RNA was extracted using the Aurum Total RNA Mini Kit according to manufacturer’s protocol (Bio-Rad, Toronto, ON). Briefly, 500 ng of total RNA was reverse transcribed with random hexamers and Super Script Reverse Transcriptase II (SSRTII; Invitrogen, Burlington, ON). The level of gene expression was quantified using QPCR and normalized to rRNA 18s. Primer sequences for CYP1A1, GREB1, and 18s were described previously by Ahmed et al. (Ahmed et al., 2009). HMOX1 mRNA was amplified with F 5’-AAGATTGCCCCAGAAAAAGCCCTGGAC-3’ and R 5’-AACTGTCGCCAACAGAAAGCTGAG-3’, NQO1 mRNA with F 5’-GGGATCCACGGGGACATGAATG-3’ and R 5’-ATTTGAATTCGGGCGTCTGCTG-3’, and NRF2 mRNA with F 5’-AGTGGATCTGCCCACTACT-3’ and R 5’-CATCTACAAACGGGAATGTCTG-3’.

RNAi-mediated knockdown of ERα and AHR

RNAi-mediated knockdown of ERα and AHR in the MCF-7 cells was performed as previously described (Ahmed et al., 2009). Briefly, MCF-7 cells were plated at a density of 300 000 cells per well in a 6-well plate and transfected with a final concentration of 50 nM of siRNA against either ERα or AHR on the following day. Cells were treated for 6 h two days after transfection. All transfection and siRNA reagents were purchased from Dharmaco, Inc., Thermo Fisher Scientific (Lafayette, CO).

Statistical Analysis

Statistical significance was determined at $p<0.05$ using the Student’s $t$-test or analysis of variance (ANOVA) with Tukey post hoc test when appropriate.
Results

**DIM induced NRF2 expression through AHR activation.**

To determine the ability of AHR and ERα to regulate NRF2 mRNA levels, we treated MCF-7 cells 10 nM E2, 10 μM SFN, 10 μM DIM alone or in combination. DIM alone and DIM+SFN treatment significantly induced NRF2 mRNA (Figure 30 A). E2 reduced but treatment with SFN or E2+SFN did not alter NRF2 mRNA levels. We recently identified and confirmed two *bona fide* AHR-bound regions at the transcriptional start site of *NRF2* induced by dioxin using ChIP-Sequencing (Lo, R., and Matthews, J., Toxicol Sci In press). In agreement with results obtained with TCDD, AHR recruitment to the regulatory region of NRF2 was induced by DIM (Figure 30 B & C). Taken together, these data revealed that DIM induced human NRF2 mRNA levels through the AHR recruitment to AHREs in the regulatory region of *NRF2* similar to that observed for murine *Nrf2*.

![Figure 30 DIM induced AHR recruitment to the upstream regulatory region of *NRF2*.](image)

A) mRNA expression of NRF2 in MCF-7 breast cancer cells treated with 0.1% DMSO vehicle control, 10 nM E2, 10 μM SFN, SFN+E2, 10 μM DIM or
DIM+SFN for 6 h. Results shown are the means ± SEM from three independent experiments. Significant differences ($p < 0.05$; ANOVA) from DMSO are indicated with asterisks (*). B) AHR and ERα recruitment to two AHR bound regions reported in our recent ChIP-Seq experiments (Lo, R., and Matthews, J., Toxicol Sci In press). Results shown are the means ± SEM from three independent experiments. Significant differences ($p < 0.05$; Student’s t-test) from DMSO are indicated with asterisks (*). Genomic locations of the two AHR-bound regions are shown using UCSC Genome Browser.

E2 repressed, whereas TCDD and DIM enhanced SFN-mediated $HMOX1$ and $NQO1$ induction in MCF-7 ERα positive breast cancer cells.

To determine the effect of AHR and ERα activation on NRF2 related genes, we treated MCF-7 ERα positive breast cancer cells with DMSO, 10 nM E2, 10 μM SFN, E2+SFN, 10 μM DIM, DIM+SFN, 10 nM TCDD, and TCDD+SFN for 6 h. The E2 concentration is within physiological levels (Gruber et al., 2002), whereas the concentrations chosen for SFN and DIM were based on their biological levels after broccoli consumption, estimated to be approximately 5 μM for SFN (Gasper et al., 2005) and 10 μM for DIM (Leong et al., 2001). The concentration of TCDD used represented a dose that induced maximum AHR transactivation (Ahmed et al., 2009; Pansoy et al., 2010).

As expected, SFN significantly induced both NQO1 and HMOX1 mRNA levels after 6 h (Figure 31 A & B). E2 repressed whereas TCDD and DIM enhanced the SFN-dependent induction of NQO1 and HMOX1 mRNA levels (Figure 31 A & B). Treatment with E2, TCDD, or DIM alone did not affect NQO1 or HMOX1 mRNA levels. To determine the selectivity of the different ligands to activate the ERα and AHR pathways, we examined their ability to induce the mRNA expression levels of Growth Regulation by Estrogen in Breast Cancer 1 (GREB1) and CYP1A1, respectively (Figure 31 C & D). TCDD specifically induced CYP1A1 expression with no effect on GREB1 expression (Figure 31 D), while E2 specifically induced GREB1 expression with no effect on CYP1A1 expression (Figure 31 C). In contrast DIM, a dual activator of AHR and ERα, induced both GREB1 and CYP1A1 expression (Figure 31 C & D). Treatment with SFN alone or in combination had no effect on CYP1A1 or GREB1 expression (Figure 31 C & D). These findings showed that even though
DIM exhibited estrogenic activity, only E2 and not DIM repressed NRF2 transactivation. The results also revealed that AHR activation by DIM or TCDD enhanced the induction of NRF2-regulated gene expression.

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**Figure 31** Treatment with DIM+SFN resulted in a non-additive increase in NQO1 and HMOX1 mRNA levels compared to SFN alone. MCF-7 cells were treated with 0.1% DMSO vehicle control, 10 nM E2, 10 μM SFN, SFN+E2, 10 μM DIM, DIM+SFN, 10 nM TCDD, and TCDD+SFN for 6 h and the mRNA expression levels for A) NQO1, B) HMOX1, C) GREB1, and D) CYP1A1 were determined as described in materials and methods. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) from DMSO are indicated with asterisks (*). Significant differences (p < 0.05; ANOVA) between SFN and the respective co-treatment groups are indicated with pound signs (#).

Knockdown of ERα reversed the repression of HMOX1 and NQO1 whereas knockdown of AHR abrogated the enhanced induction of HMOX1 and NQO1.

To confirm that the non-additive induction of NQO1 and HMOX1 was mediated through AHR, we performed RNAi-mediated knockdown of AHR and assessed the mRNA expression levels of HMOX1 and NQO1 in cells treated with SFN and SFN+DIM. AHR
mRNA and protein levels were reduced by greater than 70% with two independent siRNA sequences (Figure 32). AHR knockdown prevented the non-additive increase in NQO1 and HMOX1 expression observed in cells treated with DIM+SFN compared to SFN alone (Figure 33 A & B). Although DIM alone resulted in a weak but insignificant increase in NQO1 expression under our experimental conditions, knockdown of AHR was able to significantly reduce NQO1 mRNA levels in DIM treated cells compared to corresponding siRNA negative control (Figure 33 A).

Figure 32 RNAi-mediated knockdown of AHR and ERα in MCF-7 cells.
MCF-7 cells were transfected with non-targeting siRNA control, two independent siRNA against AHR or two independent siRNA against ERα for 48 hrs. Transfected cells were treated with 0.1% DMSO vehicle control, 10 μM SFN, 10 μM DIM, and DIM+SFN for 6 h. mRNA expression levels for AHR and ERα were determined as described in materials and methods. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) from siControl are indicated with asterisks (*). Significant
differences ($p < 0.05$; ANOVA) between the two independent siRNA are indicated with crosses (+).

![Graph](image)

**Figure 33** RNAi-mediated AHR knockdown prevented the increased NRF2-dependent gene expression after treatment with DIM+SFN compared to SFN alone.

AHR expression was knocked down transiently after 48 h of siRNA transfection. MCF-7 cells were then treated with 0.1% DMSO, 10 μM SFN, 10 μM DIM or DIM+SFN for 6 h and the mRNA expression levels for A) NQO1 and B) HMOX1 determined as described in materials and methods. Results shown are the means ± SEM from three independent experiments. Significant differences ($p < 0.05$; ANOVA) between SFN and DIM+SFN under non-targeting control siRNA conditions are indicated with asterisks (*). Significant differences ($p < 0.05$; ANOVA) from non-targeting control siRNA (siControl) within the same treatment group are indicated with asterisks (#).

In contrast to AHR knockdown, ERα knockdown did not significantly enhance SFN-induced HMOX1 or NQO1 mRNA levels. Interestingly, ERα knockdown (Figure 32) significantly increased HMOX1 mRNA expression levels in cells treated DIM+SFN.
compared to SFN alone (Figure 34 B), suggesting that under these treatment conditions, ERα represses NRF2 regulation of HMOX1. However, ERα knockdown did not further increase the enhanced expression of NQO1 in DIM+SFN treated cells, which may be due to the high endogenous NQO1 mRNA expression levels in MCF-7 cells (Jaiswal, 1994; Siegel and Ross, 2000) or reflect gene-specific differences in the ability of ERα to modulate NRF2 signaling. Taken together, these data revealed that DIM enhanced the response of NRF2 target genes via AHR activation and that in the presence of SFN+DIM, ERα was a gene specific repressor of NRF2.

![Figure 34](image)

**Figure 34** RNAi-mediated ERα knockdown differentially affected NRF2-dependent gene expression after treatment with DIM+SFN compared to SFN alone.

ERα expression was knocked down transiently after 48 h of siRNA transfection. MCF-7 cells were then treated with 0.1% DMSO vehicle control, 10 μM SFN, 10 μM DIM or DIM+SFN for 6 h and the mRNA expression levels for A) NQO1 and B) HMOX1 determined as described in materials and methods. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) between SFN and DIM+SFN under non-targeting control siRNA conditions are indicated with asterisks (*). Significant differences (p <
ERα and AHR were recruited to NRF2 binding sites in the regulatory regions of HMOX1 and NQO1 in cells treated with DIM+SFN.

To examine the mechanistic basis for ERα-mediated repression of NRF2 target genes, we performed ChIP assays to determine ERα and AHR interaction with the regulatory regions of HMOX1 and NQO1 in MCF-7 cells treated with DMSO, E2, SFN, and E2+SFN for 45 min. GREB1 enhancer region, an ERα bound and responsive region previously described by others (Carroll et al., 2005) was used as a positive control for ERα activation. As expected, E2 caused a significant increase in ERα recruitment to the GREB1 enhancer, which was not affected by SFN (data not shown). SFN significantly increased NRF2 recruitment to the AREs of both HMOX1 and NQO1 at 45 min, which was unaffected by E2 co-treatment (Figure 35 A & D). Surprisingly, ERα recruitment to HMOX1 and NQO1 was detected only in cells treated with E2+SFN (Figure 35 B & E). AHR was not recruited to HMOX1 or NQO1 under any treatment conditions examined (Figure 35 C & F).
Figure 35 Ligand-dependent recruitment of NRF2, ERα and AHR to NQO1, and HMOX1 in MCF-7 cells treated with E2, SFN, or E2+SFN.

ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 nM E2, 10 μM SFN or SFN+E2 for 45 min to determine (A & D) NRF2, (B & E) ERα, (C & F) AHR recruitment to NQO1 and HMOX1. The genomic locations of the primers relative to TSS are shown in the schematics below the graphs. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) from DMSO are indicated with asterisks (*).

We next examined the mechanism by which TCDD might enhance NQO1 and HMOX1 expression. We conducted the ChIP assays to determine ERα and AHR interaction with the regulatory regions of HMOX1 and NQO1 in MCF-7 cells treated with DMSO, TCDD, SFN, and TCDD+SFN for 45 min. The CYP1A1 AHRE cluster was used as a positive control for AHR activation. As expected, TCDD caused a significant increase in AHR recruitment to CYP1A1, which was not affected by SFN (data not shown). SFN significantly increased NRF2 recruitment to HMOX1 and NQO1, which was not affected by TCDD (Figure 36 A & D). TCDD alone resulted in AHR recruitment to NQO1 but not
HMOX1. On the other hand, TCDD+SFN resulted in AHR recruitment to both HMOX1 and NQO1 (Figure 36 C & F). This is consistent with our hypothesis that AHR might be involved in enhancing the expression of those two genes. ERα recruitment was not increased above vehicle DMSO control levels for any of the treatment conditions examined (Figure 36 B & E).

Figure 36 Ligand-dependent recruitment of NRF2, ERα, and AHR to NQO1, and HMOX1 in MCF-7 cells treated with TCDD, SFN, or TCDD+SFN.

ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 nM TCDD, 10 μM SFN or TCDD+SFN for 45 min to determine (A & D) NRF2, (B & E) ERα, (C & F) AHR recruitment to NQO1 and HMOX1. The genomic locations of the primers relative to TSS are shown in the schematics below the graphs. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) from DMSO are indicated with asterisks (*).

Since DIM is a dual activator of both AHR and ERα, we were interested to determine how DIM would affect the binding patterns of AHR and ERα to NQO1 and HMOX1 using ChIP assays. In agreement with its ability to activate both the AHR and the estrogen...
signaling pathways, DIM induced ERα and AHR recruitment to the GREB1 and the CYP1A1, respectively (data not known). SFN significantly increased NRF2 recruitment to both NQO1 and HMOX1, which was unaffected by DIM co-treatment (Figure 37 A & D). DIM treatment alone resulted in increased AHR recruitment to NQO1 but not HMOX1 (Figure 37 C & F). This was in agreement with the fact that NQO1, but not HMOX1, was part of the AHR gene battery (Yeager et al., 2009). Interestingly, recruitment of both ERα and AHR to HMOX1 and NQO1 was only observed in cells co-treated with DIM+SFN (Figure 37).

Figure 37 Ligand-dependent recruitment of NRF2, ERα and AHR to NQO1, and HMOX1 in MCF-7 cells treated with DIM, SFN, or DIM+SFN. ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 μM DIM, 10 μM SFN or SFN+DIM for 45 min to determine (A & D) NRF2, (B & E) ERα, (C & F) AHR recruitment to NQO1 and HMOX1. Results shown are the means ± SEM from three independent experiments. Significant differences (p < 0.05; ANOVA) from DMSO are indicated with asterisks (*).
AHR-mediated enhancement and ERα-mediated repression of NQO1 and HMOX1 expression might be associated with changes in Histone H3 Lysine 9 acetylation

To investigate potential epigenetic factors that might govern ERα-dependent repression of NRF2 transactivation, we examined the level of Histone H3 Lysine 9 acetylation (H3K9Ac) in MCF-7 cells treated with E2, SFN, and E2+SFN at HMOX1 and NQO1. Histone acetylation was normalized to total Histone H3 (H3K9Ac:H3 ratio) to account for potential changes in histone H3 density. In agreement with SFN-dependent increases in NQO1 and HMOX1 mRNA levels, SFN treatment increased the level of H3K9Ac:H3 at both HMOX1 and NQO1 (Figure 38 A & C). SFN-dependent increases in H3K9Ac:H3 at HMOX1 and NQO1 were prevented with SFN+E2 co-treatment, consistent with E2-dependent repression of SFN activated NRF2 shown in Figure 31.

Figure 38 E2 prevented SFN-dependent increased histone H3 Lysine 9 acetylation and p300 recruitment to NQO1 and HMOX1.

ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 nM E2, 10 μM SFN, and SFN+E2 for 45 min to determine changes in H3K9Ac (A & C) and the recruitment of the histone acetyltransferase, p300 (B & D) to NQO1 and HMOX1. H3K9Ac levels are normalized to total Histone H3 present and results shown are the means ± SEM from three independent experiments. Results for p300 recruitment are the means ± SEM from three
independent experiments. Significant differences \((p < 0.05; \text{ANOVA})\) from DMSO are indicated with asterisks (*).

We next sought to identify co-regulators that might be responsible for the decreased H3K9Ac:H3 levels observed at \(NQO1\) and \(HMOX1\) after co-treatment of SFN+E2 compared to SFN alone. To this end, we screened for SFN- and SFN+E2-dependent recruitment of several co-regulators to both genes. The histone deacetylases (HDACs; HDAC1, HDAC4/5/7 and SIRT1), nuclear receptor corepressor (NCoR), and nuclear receptor co-activators 1-3 showed no clear recruitment patterns to \(NQO1\) and \(HMOX1\) that were consistent with E2-mediated repression of both genes (data not shown). We observed, however, that SFN treatment resulted in increased p300 recruitment to \(NQO1\) and \(HMOX1\), which was significantly reduced in cells co-treated with SFN+E2 (Figure 38 B & D). These findings suggested that the E2-dependent decrease in H3K9Ac:H3 at \(NQO1\) and \(HMOX1\) was associated with increased ER\(\alpha\) but decreased p300 recruitment to both genes.

The levels of H3K9Ac and recruitment of p300 to \(NQO1\) and \(HMOX1\) were also determined in MCF-7 cells treated with TCDD, DIM, SFN, TCDD+SFN or DIM+SFN. TCDD (Figure 39A & C) alone increased H3K9Ac:H3 at \(NQO1\) but not \(HMOX1\), which was consistent with the fact that NQO1 but not HMOX1 is an AHR target. Similarly, DIM alone led to a trend for an increase in H3K9Ac:H3 at NQO1 but not at HMOX1 (Figure 40A & C). Despite the observation that TCDD+SFN and DIM+SFN enhanced the expression of NQO1 and HMOX1 (Figure 31), neither TCDD+SFN (Figure 39 B & D) nor DIM+SFN (Figure 40 B & D) enhanced H3K9Ac:H3 or increased the recruitment of p300 compared to SFN alone. The presence of AHR at \(NQO1\) and \(HMOX1\) after co-treatment with DIM+SFN might have prevented the inhibitory action of ER\(\alpha\) on histone acetylation and p300 recruitment to both genes. Taken together, these data suggest that under conditions when both AHR and ER\(\alpha\) are activated, AHR blocks the repressive action of ER\(\alpha\) on NRF2 transactivation.
Figure 39 TCDD did not affect SFN-dependent increased histone H3 Lysine 9 acetylation and p300 recruitment to *NQO1* and *HMOX1*.

ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 nM TCDD, 10 μM SFN, and TCDD+SFN for 45 min to determine changes in H3K9Ac (A & C) and the recruitment of the histone acetyltransferase, p300 (B & D) to *NQO1* and *HMOX1*. H3K9Ac levels are normalized to total Histone H3 present and results shown are the means ± SEM from three independent experiments. Results for p300 recruitment are the means ± SEM from three independent experiments. Significant differences (*p* < 0.05; ANOVA) from DMSO are indicated with asterisks (*).
Figure 40 DIM did not affect SFN-dependent increased histone H3 Lysine 9 acetylation and p300 recruitment to \textit{NQO1} and \textit{HMOX1}.

ChIP assays were performed on MCF-7 cells treated with 0.1% DMSO vehicle control, 10 μM DIM, 10 μM SFN, and DIM+SFN for 45 min to determine changes in H3K9Ac (A & C) and the recruitment of the histone acetyltransferase, p300 (B & D) to \textit{NQO1} and \textit{HMOX1}. H3K9Ac levels are normalized to total Histone H3 present and results shown are the means ± SEM from three independent experiments. Results for p300 recruitment are the means ± SEM from three independent experiments. Significant differences (\(p < 0.05\); ANOVA) from DMSO are indicated with asterisks (*).
Discussion

Dietary consumption of cruciferous vegetables results in exposure to many chemopreventative agents, including SFN and DIM (Hauder et al., 2011). Exposure to these two compounds results in the simultaneous activation of NRF2, AHR and ERα signaling pathways. SFN activation of NRF2 and DIM activation of AHR have been shown to inhibit breast cancer cell growth (Prud'homme et al., 2010; Zhang et al., 2009). AHR regulates NRF2 expression levels; however, E2-activated ERα represses NRF2 activity. In light of the importance of AHR and NRF2 in mediating Phase I and II drug metabolism and the critical role of ERα in breast carcinogenesis, a better understanding of the crosstalk among all three receptors should improve therapeutic response and decrease potential adverse drug-drug interactions. Our data reveal a complex signaling interplay among NRF2, AHR and ERα after SFN+DIM treatment at the NRF2 regulated target genes, NQO1 and HMOX1.

We report that, similar to murine Ahr, human AHR is a key regulator of NRF2 and the corresponding increase in NRF2 target gene expression after co-treatment with SFN+DIM. AHR regulates the murine Nrf2 by binding to the AHREs located proximal to the Nrf2 promoter (Miao et al., 2005). A recent study reported that Nqo1 was up-regulated by TCDD in wild-type mice, but not in Nrf2-null mice, suggesting that Nrf2 was required for Ahr-dependent induction of Nqo1 in the murine system (Yeager et al., 2009). They also found that NRF2 was required for the TCDD-mediated and AHR-dependent induction of TCDD responsive UDP-glucuronosyltransferase and glutathione-s-transferase isoforms, revealing extensive functional overlap between the AHR and NRF2 signaling pathways (Yeager et al., 2009). The authors proposed two possible mechanisms by which TCDD may induce gene expression changes via the AHR-Nrf2 pathway: (1) TCDD induces cytochrome P450 levels causing an increase in oxidative stress, and the activation of Nrf2; and (2) Ahr binds to AHREs in the regulatory region of Nrf2, thereby increasing the levels of Nrf2. The data presented here and in our recent ChIP-Seq study where we described two AHR-bound regions at the transcription start site of NRF2 after treatment with TCDD (Lo, R., and Matthews, J., Toxicol Sci In press), provide support for direct regulation of NRF2 levels by AHR in human cell lines. In the absence of SFN or inhibition of KEAP1, AHR-mediated increase in NRF2 levels might not have much of an effect due to continuous KEAP1-
mediated NRF2 degradation. However, inhibition of KEAP1 results in the nuclear accumulation of \textit{de novo} synthesized NRF2 protein, increasing target gene expression (Kobayashi et al., 2006). Simultaneous activation of AHR and NRF2 following co-exposure to SFN+DIM induces a greater than additive increase in NRF2 transactivation through inhibition of KEAP1 coupled with the AHR-dependent increase in \textit{de novo} NRF2 expression levels. In agreement with our data, a previous study also reported synergistic induction of NQO1 mRNA expression after treatment with SFN+DIM compared to SFN alone, showing that maximum NQO1 up-regulation requires both AHR and NRF2 activation (Saw et al., 2011). In support of this notion, pretreatment of human colon LS-174 and Caco-2 adenocarcinoma cell lines with SFN+ICZ, an indole and potent AHR activator (Wihlen et al., 2009), conferred greater protection against DNA damage than did either phytochemical alone (Bonnesen et al., 2001). This suggests that combinations of indoles and isothiocyanates result in greater health benefit than the use of individual compounds, perhaps due to the increased NRF2 response.

Interaction between AHR and NRF2 signaling can also vary with cellular context. NRF2 activators such as SFN and other isothiocyanates inhibited AHR nuclear translocation in MCF-7 cells (Skupinska et al., 2009) and AHR activation in rat liver slices (Abdull Razis et al., 2012a), whereas SFN was a weak inducer of CYP1A1 through AHR activation in mouse and human liver cell lines (Anwar-Mohamed and El-Kadi, 2009). However, we did not observe any affects of SFN on AHR transactivation in the present study.

Reciprocal crosstalk between ER\textsubscript{α} and NRF2 has been reported where ER\textsubscript{α} represses NRF2 transactivation (Montano et al., 2004), while SFN down-regulates ER\textsubscript{α} mRNA and protein levels (Hu et al., 2004; Ramirez and Singletary, 2009) suggesting that the inhibitory crosstalk action of ER\textsubscript{α} on NRF2 transactivation may be short lived and transient in nature. In support of these studies, time-course ChIP experiments revealed that ER\textsubscript{α} is recruited to \textit{NQO1} and \textit{HMOXI} in SFN+E2 treated cells for at least 6 h but not 24 h after treatment (data not shown).

DIM indirectly activates both ER\textsubscript{α} (Leong et al., 2004b) and ER\textsubscript{β} (Vivar et al., 2010), although a recent study reported it to be a ER\textsubscript{β}-selective activator (Lo and Matthews,
Little is known about the influence of ERβ on NRF2 signaling, but since ERβ represses the actions of ERα (Matthews et al., 2006) high levels of ERβ might block the ability of ERα to repress NRF2. The influence of ERβ on the DIM-mediated response was not evaluated due to the very low levels of ERβ in the MCF-7 cells used in this study (data not shown). Since E2-activated ERα inhibits the NRF2 signaling pathway, DIM-activated ERα may offset any benefits from SFN-mediated NRF2 activation. However in the presence of AHR, DIM activates AHR, causing its recruitment to \( NQO1 \) and \( HMOX1 \) and counteracting the ERα-dependent repression of both genes by preventing the ERα-dependent removal of the p300 and decrease in K9AcH3 levels. These findings support the view that dietary exposure to SFN and DIM would prevent the inhibitory actions of ERα on NRF2 signaling in tissues that expression NFR2, AHR, and ERα.

Taken together, the data present here and in other reports have led us to propose an intricate interaction network where the three receptor pathways (AHR, ERα, and NRF2) are so functionally linked that a slight modulation, due to ligand activation, in one system can have significant consequences on the other two pathways. In the presence of SFN, NRF2 is recruitment to AREs in the genome to regulate gene expression. ERα when activated prevents the recruitment of p300, histone acetylation and NRF2-dependent gene expression. AHR increases NRF2 mRNA levels but also prevents the inhibitory effect of ERα on NRF2 transactivation by maintaining p300 recruitment and enhancing NRF2 transactivation. These findings have important implication for hormone and oxidative stress related diseases such as breast cancer in which all three receptors have been implicated in this disease.
Significance of Study

Our data revealed a complex functional interplay among the NRF2, AHR, and ERα signaling pathways upon exposure to dietary compounds that can simultaneously activate all three receptor systems. ERα repressed whereas AHR enhanced NRF2-dependent expression of NQO1 and HMOX1 by altering the recruitment of p300 and Histone H3 lysine 9 acetylation. Enhanced NRF2 transcriptional activity through AHR activation might have important biological implications in the production of genotoxic estrogens and breast cancer development.
Chapter 6 Discussion

6.1 General Discussion

There are two co-existing mechanisms of estrogen dependent breast cancer development. Estrogen can act as a mitogen to increase cell proliferation or it can be metabolized into genotoxic metabolites that cause mutation and tumor formation. This thesis aimed to elucidate the regulation of Phase I and II genes involved in the bioactivation and the detoxification of genotoxic estrogen metabolites by ERα, AHR, and NRF2 in the development of breast cancer.

Prior to our promoter-focused analysis of ERα binding sites in T-47D breast cancer cells, the genomic binding pattern of ERα was described only in the MCF-7 breast cancer cell line, which might have cell-line specific artifact and might overlook important ERα binding targets in other cell lines and/or primary mammary epithelial cells. Comparison between our ERα binding sites in T-47D cells and those by Carroll et al. (Carroll et al., 2006) in MCF-7 cells revealed a modest overlap between the two cell lines. Interestingly, the comparison also revealed that CYP2B6, a Phase I bioactivation enzyme, was differentially regulated by ERα in the two cell lines. In addition to CYP2B6, studies by various groups have demonstrated that ERα can up-regulate other CYP450s including CYP1A1, CYP1B1, and CYP2A6 either directly or indirectly (Higashi et al., 2007; Matthews et al., 2005; Tsuchiya et al., 2004). Estrogen-dependent and ERα–mediated regulation of Phase I enzymes might increase the generation of reactive metabolites in the breast tissue, and thereby might have implications in the genotoxic metabolite pathway of breast cancer development.

Another receptor that has been long established as one of the primary regulator of Phase I bioactivation was AHR. Recent microarray studies have indicated that AHR might regulate the expression of genes involved in biological pathways in addition to xenobiotic metabolism (Boverhof et al., 2005). However, it remains unclear whether those effects are mediated directly by the genomic activation of AHR (ie. direct AHR-DNA interaction) or the secondary effect of AHR activation. Only a few studies have investigated the genomic
binding profile of AHR (Ahmed et al., 2009; Pansoy et al., 2010; Sartor et al., 2009), and none have investigated genomic AHR binding \textit{in vivo}. Our promoter-focused analysis of AHR binding sites revealed differential binding pattern of AHR \textit{in vivo} compared to that \textit{in vitro}. Some AHR binding sites \textit{in vivo} were not associated with gene regulation, indicating additional epigenetic mechanisms regulating the expression AHR target genes. We determined that Histone H3 lysine 9 acetylation (H3K9Ac) was associated with AHR-dependent up-regulation of \textit{cyp1b1}, \textit{nqo1}, and \textit{nrf2} \textit{in vivo}, whereas histone H3 lysine 4 dimethylation (H3K4Me2) was not. CYP1B1, NQO1, and NRF2 are all involved in the bioactivation and detoxification of estrogen, thereby linking AHR to genotoxic pathway of breast cancer development.

Our initial analysis of AHR binding sites was performed in mouse hepatic tissue since the liver was the primary target of AHR activation. In Chapter 4, we extended our study to MCF-7 breast cancer cells to investigate similarities and differences in AHR signalling in breast cancer using a high throughput, sequencing based approach. Many aspects of AHR signalling were similar between mouse hepatic tissue and MCF-7 breast cancer cells including binding sequences, interacting transcription factors, and even target genes. Our study was the first sequencing based identification of AHR and ARNT binding sites in breast cancer, further advancing our understanding of AHR signalling and the mechanism of Phase I regulation in breast cancer.

Lastly, an increase in bioactivation and the generation of reactive metabolites can be countered with an increase in NRF2-dependent expression of Phase II detoxification enzymes. Interestingly, ER\(\alpha\) and AHR have been reported to exert opposite effects on NRF2 activity with ER\(\alpha\) having a negative and AHR having a positive effect. However, it remains unclear which receptor dominates when both ER\(\alpha\) and AHR signalling pathways were simultaneously activated in the presence of a dual activator such as DIM. DIM is the primary metabolite of IC3, which is found at high concentrations in cruciferous vegetables along with isothiocyanates such as SFN (prototypical NRF2 activator). Therefore, simultaneous activation of ER\(\alpha\), AHR, and NRF2 is a likely scenario from our regular diet. Our study in Chapter 5 indicated that although ER\(\alpha\) and AHR were both recruited to NRF2 binding sites, AHR was able to override the inhibitory effect of ER\(\alpha\) on NRF2 to enhance NRF2-
dependent gene transactivation. AHR-dependent and ERα-dependent modulation of NRF2 activity and Phase II gene expression was regulated by epigenetic factors such as p300 recruitment and H3K9Ac.

In summary, this thesis investigated the role of three transcription factors – ERα, AHR, and NRF2 - in modulating genes involved in the bioactivation and detoxification pathways of breast cancer. They might represent potential therapeutic targets for the prevention of breast cancer.

### 6.2 E2-dependent regulation of CYP2B6

ERα has been reported to regulate several CYP450s such as CYP1A1, CYP1B1, and CYP2A6 through different mechanisms. ERα-dependent induction of CYP1B1 and CYP2A6 was mediated through direct interaction with DNA, while ERα-dependent induction of CYP1A1 was indirect, mediated through interaction with AHR. Previous studies have indicated a clear association between estrogen signalling pathway and the expression of CYP2B6. High concentration E2 exposure induced Cyp2b10 (the mouse orthologue of the human CYP2B6) in culture (Kawamoto et al., 2000; Koh et al., 2012). ERα-positive breast tumors had higher CYP2B6 expression (Tozlu et al., 2006). Lastly, CYP2B6 expression and activity was higher in the female gender (Lamba et al., 2003). However, the precise mechanism has not been fully elucidated prior to our present study. In Chapter 2, I have provided several lines of evidence that E2 induces CYP2B6 through the classical genomic pathway of estrogen signalling. 1) ChIP assays demonstrated direct ERα-DNA and NCoA3-DNA interaction at two regulatory regions adjacent to the CYP2B6 and CYP2B7 promoters. 2) Mutation analysis of the binding sites revealed the requirement of an ERE. 3) An estrogen receptor degrader, fulvestrant (ICI 182,780), significantly down-regulated E2-induced CYP2B6 expression. 4) RNAi knockdown of ERα significantly down-regulated CYP2B6 expression. However, CYP2B6 was differentially regulated in two estrogen dependent breast cancer cell lines, suggesting other important factors governing CYP2B6 expression in addition to ERα.
There are at least two proposed mechanisms by which E2 regulates CYP2B6 expression that are different from that described in Chapter 2. The first proposed mechanism is through the activation of the constitutive androstane receptor (CAR; NR1I3) and the second proposed mechanism is through ERα-AP1 interaction. CAR interacts with the Phenobarbital response enhancer module (PBREM) upstream of CYP2B6 to induce its expression. CAR is a key regulator of xenobiotic metabolism and responds to a wide variety of ligands including phenobarbital, androstanol, TCPOBOP, CITCO, and progesterone (Swales and Negishi, 2004). In 2000, Kawamoto et al. reported estrogen dependent activation of CAR, linking estrogen to xenobiotic/steroid metabolism (Kawamoto et al., 2000). The concentration of estrogen (10 μM) required to induce Cyp2b10 expression in that study was substantially greater than the physiological concentration (picomolar to nanomolar range) (Kawamoto et al., 2000). Moreover, pharmaceutical estrogens such as diethylstilbestrol failed to enhance CAR activity (Kawamoto et al., 2000). Based on these findings alone, the authors concluded that estrogen activation of CAR and subsequent Cyp2b10 induction might involve a mechanism independent of the estrogen receptor (Kawamoto et al., 2000). There are many explanations that might address the discrepancies between Kawamoto et al. and our studies presented in Chapter 2. Firstly, Kawamoto et al. investigated estrogen activation of CAR in human HepG2 cells over-expressing the mouse CAR (Kawamoto et al., 2000). However, HepG2 cells do not express ERα (Barkhem et al., 1997). Hence, Kawamoto et al. simply did not have sufficient evidence to suggest that ERα was not involved in estrogen-dependent induction of Cyp2b10. Secondly, primary mouse hepatocytes were employed in their experiments. The expression status of ERα in those primary hepatocytes was a confounding variable, which was not controlled in any of the experiments. Thirdly, the ERE responsible for ERα-dependent regulation of human CYP2B6 is not conserved in the mouse Cyp2b10 regulatory region, suggesting that ERα-dependent regulation of CYP2B6 might be species specific (Lo et al., 2010). Lastly, the use of supra-physiological concentration of E2 had minimal biological relevance as E2 concentration will never reach micromolar concentration under normal conditions (Gruber et al., 2002). In summary, the experiments presented by Kawamoto et al. were not designed to elucidate ERα-dependent regulation of CYP2B6 and had minimal biological relevance.
Our study and a recently published paper by Koh et al., on the other hand, clearly demonstrated the involvement of ERα in the regulation of CYP2B6, although our conclusions on the precise mechanism of regulation differed slightly (Koh et al., 2012; Lo et al., 2010). Koh et al. described a tethering mechanism in which ERα did not interact directly with DNA, but rather interacted with the AP-1 transcription complex at two AP-1 binding sites located -1782/-1776 and -1664/-1658 from CYP2B6 (Koh et al., 2012). This conclusion was supported by the fact that a DNA binding deficient mutant of ERα was able to activate a reporter gene regulated by a ~3 kb region immediately upstream of CYP2B6 and that mutation of the two AP-1 binding sites attenuated E2-induced reporter gene activity (Koh et al., 2012). Although we arrived at different conclusions, our results were in fact similar. One of the AP-1 binding sites, which they labeled A2 overlaps with our ERE (5’-CTGGGTCAAA-3’; ERE shown in bold, AP-1 underlined). The other AP-1 binding sites, A1, was unfortunately not cloned into our pGL3-CYP2B6 luciferase reporter vector. In their study, single mutation of either AP-1 binding sites was not sufficient to attenuate reporter gene activity, whereas double mutation was (Koh et al., 2012). Since our pGL3-CYP2B6 luciferase reporter vector already lacked the A1 AP-1 site, a point mutation of the ERE (which overlaps with the A2 AP-1) effectively created a similar luciferase construct containing a double AP-1 mutation. This explains the “discrepancy” as to why a single mutation at one AP-1 site in our construct completely abrogated estrogen induced luciferase activity in our study. A common observation between our study and theirs was that the A2 AP-1 or ERE site is an important but not a required enhancer element in E2 induced CYP2B6 expression.

Another discrepancy between our studies was that our ERα mutant containing two amino acids substitution (E204A and G205A) in the DBD was unable to induce the luciferase activity of pGL3-CYP2B6. Our results therefore suggested that ERα was interacting directly with the regulatory region of CYP2B6 through its DBD. On the other hand, Koh et al. used a different ERα mutant which contained three nucleotides substitution within the DBD (Harnish et al., 1998; Koh et al., 2012; Mader et al., 1989). Although the ERα mutant was unable to activate an ERE-regulated reporter, their ERα mutant activated the glucocorticoid response element (GRE) (Harnish et al., 1998; Mader et al., 1989). It was
unclear why they chose to examine an ER\(\alpha\) mutant with the ability to bind GRE. The mutant’s ability to activate the GRE represented a confounding variable that might contribute to the discrepancies between their studies and ours.

AP-1 is a heterodimeric transcription complex, consisting of c-fos and c-jun. Koh et al. demonstrated AP-1 interaction with \(A1\) AP-1 binding site using the electrophoretic mobility shift assay (Koh et al., 2012). There are multiple commonly used assays to investigate DNA-protein interaction including reporter gene assay, electrophoretic mobility shift assay (EMSA) and ChIP. One of the weaknesses of the EMSA is that DNA-protein interaction is based on a radiolabelled probe \textit{in vitro} instead of genomic DNA in its native chromatic structure in intact cells. Quantifying the level of interaction relies on qualitative measurements such as band intensity and shift rather than quantitative approaches such as qPCR for ChIP. Furthermore, EMSA results are sometimes difficult to interpret as more than one electrophoretic outcome can indicate DNA-protein interaction including a band super-shift in the presence or absence of an antibody and a decrease in band intensity with the addition of antibody. Interestingly, Koh et al., despite using the ChIP assay to demonstrate ER\(\alpha\)-DNA interaction, chose to use the EMSA to demonstrate c-fos and c-jun interaction. The quality of their EMSA gel was suggestive at best and did not convincingly demonstrate c-fos and c-jun recruitment to \(A1\) AP-1 binding site. Furthermore, AP-1 loss-of-function experiments such as the over-expression of dominant negative variants of c-fos or c-jun or incubation with AP-1 small molecule inhibitors, were required to demonstrate the involvement of the AP-1 complex in the regulation of CYP2B6.

### 6.3 CYP2B6 and breast cancer

CYP2B6 accounts for <10% of total hepatic CYP expression, but is ubiquitously expressed with detectable levels in intestine, kidney, lung, skin, brain and mammary gland (Ding and Kaminsky, 2003; Janmohamed et al., 2001; Miksys et al., 2003). CYP2B6 contributes to the bioactivation of a wide variety of pharmaceutical agents including cyclophosphamide, buproprion and tamoxifen; environmental contaminants such as aflatoxin
B and dibenzanthracene; and nicotine and methylenedioxyamphetamine (MDMA “ecstasy”) (Hodgson and Rose, 2007; Wang and Tompkins, 2008). Tamoxifen is the common first-line of therapy against ER-positive breast cancer. Tamoxifen is a pro-drug that requires enzymatic C₄ hydroxylation for full efficacy. Activation of tamoxifen is mediated primarily by CYP2D6, which is a very polymorphic CYP. Although controversial, it is conceivable that in CYP2D6 poor metabolizers, CYP2B6 might have a minor role in metabolizing tamoxifen into its active form, Z-4-hydroxytamoxifen (Boocock et al., 2002; Coller et al., 2002). Consistent with the idea that CYP2B6 is associated with positive prognosis for hormonal dependent breast cancer, relapse free survival was modestly correlated with CYP2B6 over-expression ($p = 0.078$). Post-menopausal ER-positive breast cancer patients with high levels of CYP2B6 experienced higher relapse free survival rates compared to moderate and low levels of CYP2B6 (Bieche et al., 2004). However, the relationship between CYP2B6 and favorable prognosis was not clear.

### 6.4 ERα binding sites in T-47D breast cancer cells

In our analysis, we identified CYP2B6 to be one of the many differentially regulated genes in the two cell lines. We identified in total 243 ERα binding sites, which was substantially lower than the 3,665 ERα binding sites in MCF-7 cells. Furthermore, we only observed a modest 36% overlap between the two datasets. There are many explanations for the discrepancy including differences in 1) sample preparation, 2) hybridization microarray platform (promoter-focused vs. genome-wide), 3) the concentration of estrogen exposure, and 4) cell line specific estrogen responsiveness. Hurtado et al. in 2011 conducted a full comparison between ERα binding sites in T-47D and MCF-7 and reached similar conclusions. Overlap between ERα binding sites (more specifically FOXA1-ERα binding sites) in MCF-7 and T-47D remains around 29-30% with T-47D being substantially less estrogen responsive (1,716 binding sites in T-47D compared to 14,059 binding sites in MCF-7) (Hurtado et al., 2011). Collectively, this emphasized the importance of examining multiple breast cancer cell lines to produce an accurate representation of estrogen signalling in breast cancer.
With respect to the sequences of the ERα binding sites, the ERE (5’AGGTCAnnnTGACCT3’) was significantly enriched as expected. Other enriched transcription factor binding sites included Myf and Hand1-Tcfe2a, which were identified as positive hits likely due to the fact that they both contained a half site ERE (5’AGGTCA3’). In addition, binding sequence enrichment analysis also identified the binding sequences for other transcription factors such as foxd3 (5’TGTTTTAC-3’) and FOXI1 (5’TGTTTTAT3’). Both had binding sequences similar to that of FOXA1 (5’TGTTTACTTTG3’) (Stormo, 2000), the pioneer factor governing ERα-DNA interaction. This suggested that forkhead proteins are major determinants of estrogen signalling in T-47D as well as MCF-7, a finding verified by Hurtado et al. (Hurtado et al., 2011).

One of the weaknesses of our ChIP-chip study was the coverage of our microarray platforms that were limited to the region approximately 10 kb surrounding annotated transcription start sites (TSS). Multiple genome-wide ERα binding site analysis in MCF-7 cells have indicated that many ERα binding sites were located distal to annotated TSS (Carroll et al., 2005; Carroll et al., 2006; Lin et al., 2007). Distal ERα binding sites regulated the expression of their target genes thousands of kilobases away by forming intrachromosomal looping structures (Figure 41) (Li et al., 2010). Target genes were differentially regulated depending on their locations relative to the looping structures in MCF-7 cells (Li et al., 2010). For example, genes immediately adjacent to the ERα binding sites (ie. anchoring genes) were associated with gene activation (Li et al., 2010). On the other hand, genes inside the looping structures (ie. looping genes) were associated with gene repression (Li et al., 2010). Unfortunately, our promoter-focused ChIP-chip study in T-47D was not designed to investigate intrachromosomal interaction. It remains unclear whether these chromatin looping structures also exist in T-47D cells.
Figure 41 Gene regulation by distal binding sites via DNA looping mechanism.

Target genes were differentially regulated depending on their locations relative to the looping structure in MCF-7 breast cancer cells. Anchoring genes adjacent to binding sites were usually up-regulated, whereas looping genes located in the looping structures were usually repressed.

6.5 Cell line differences between T-47D and MCF-7 cells

T-47D and MCF-7 are two cell lines commonly used to investigate estrogen signalling in breast cancer, as both cell lines expressed a relatively high level of ERα protein. In addition to CYP2B6 which was differentially regulated in the two cell lines, we have demonstrated in Chapter 2 that the expression levels of two commonly examined estrogen target genes, TFF1 and GREB1, were also significantly different between T-47D and MCF-7. TFF-1 mRNA was expressed at a much higher level in both vehicle-treated and E2-treated MCF-7 cells compared to T-47D cells, while E2-induced GREB1 induction was more robust in MCF-7 cells (Lo et al., 2010). The expression differences were associated with differential ERα and NCoA3 recruitment for both genes. While it was established that the ERα binding patterns were substantially different between the two cell lines (~70% difference) (Hurtado et al., 2011; Lo et al., 2010), the molecular mechanism for the differences remains unclear and
might involve epigenetic factors such as histone methylation. For example, transient knockdown of an insulator protein CTCF can rescue ERα recruitment to regions that were previously not bound by ERα in ZR-75 breast cancer cells (Hurtado et al., 2011). The new ERα binding sites induced by CTCF knockdown were associated with an increase in Histone H3 lysine 4 monomethylation and an increase in FOXA1 recruitment (Hurtado et al., 2011), highlighting the importance of other epigenetic and co-regulatory factors.

6.6 ChIP-chip: AHR binding sites in hepatic tissues

Many studies have investigated gene expression profile in animals treated with TCDD; however, only a few have utilized ChIP-chip to characterize the primary effect of AHR activation by mapping genome-wide AHR-DNA interaction. These studies were limited to biological models such as mouse hepatoma cells, activated mouse B-cell lymphoma cells, and human breast cancer cells (Ahmed et al., 2009; De Abrew et al., 2010; Sartor et al., 2009). As one of the primary target tissues of AHR activation is the liver (Bock and Kohle, 2006), we provided an analysis of promoter-focused AHR binding sites in vivo in hepatic tissue in Chapter 3 to help increase our understanding in AHR signalling and the regulation of xenobiotic metabolism. We followed up that experiment with a complete genome-wide analysis of AHR binding sites in mouse hepatic tissue.

We identified a total of 1,642 and 508 AHR binding sites at 2 and 24 h. The temporal discrepancy in terms of the numbers of AHR binding sites was intriguing and might be attributed to several factors. For example, the greater number of AHR binding sites after 2 h of treatment might represent a strong initial response to TCDD that correlated with a substantial increase in the number of differentially regulated genes at 4 h (Boverhof et al., 2005; Dere et al., 2006). On the other hand, the lower number of AHR binding sites at 24 h represented persistent AHR targets since the majority of the binding sites at 24 h (430/508, 85%) were also bound by AHR at the earlier time point. The temporal discrepancy might also be explained by negative regulatory pathways that control AHR signaling. AHR-dependent expression of AHR repressor (AHRR) might 1) compete with AHR for ARNT
heterodimerization or 2) AHRR-ARNT heterodimers might compete with AHR-ARNT for AHRE interaction (Hahn et al., 2009; Mimura et al., 1999). Although the precise mechanism of action was controversial (Evans et al., 2008), both scenarios involving AHRR would lead to a decrease AHR-ARNT-AHRE interaction observed at 24 h. Another well-established mechanism that might control AHR signalling was TCDD-dependent degradation of AHR through the 26S proteosomal pathways (Ma and Baldwin, 2000; Pollenz, 2002). However, the degree of TCDD-dependent AHR degradation varied depending on the experimental conditions (Pollenz, 2002). In Hepa1c1c7 cells, AHR protein level was reduced to <5% within 24 h of TCDD treatment for as long as 72 h (Giannone et al., 1998). In Sprague-Dawley rats, 10 $\mu$g/kg dose of TCDD for 24 h resulted in only a 70% decrease in AHR protein levels, which recovered to basal levels after 168 h (Pollenz et al., 1998). Lastly, the temporal discrepancy might stem from the difference in quality between datasets from the two time points. Both datasets were analyzed at a statistical cutoff of false detection rate 1% (FDR1). FDR can be influenced by the robustness of the response, consistency between biological replicates, and technical errors during sample preparation and hybridization process. Fewer regions made the cutoff of FDR1 at 24 h compared to 2 h, which might indicate a lower degree of consistency and robustness in the 24 h dataset. Regardless of the precise explanation for the temporal discrepancy, our ChIP-chip experiments after 2 and 24 h TCDD treatment demonstrated persistent activation of AHR in vivo. This persistent deregulation of AHR target genes might be involved in some of the toxic endpoints associated with TCDD toxicity.

With respect to the DNA sequences of the AHR binding sites, the core AHRE (5′GCGTG3′) was significantly enriched as expected. Promoter regions are GC-rich and might bias the probability of random AHREs appearing in our promoter-focused dataset. Interestingly, the same core AHRE was enriched in our genome-wide AHR ChIP-chip experiments in hepatic tissues of immature female mice (Dere et al., 2011). Enriched sequences common between the promoter-focused (Lo et al., 2011) and the genome-wide (Dere et al., 2011) ChIP-chip studies included the binding motifs for GC-box factor SP1, hypoxia inducible factor (HIF, bHLH/PAS) family, E2F-Myc activator, nuclear respiratory factor 1, nuclear receptor subfamily 2 factor (NR2F), and early growth response factor
(EGRF). The enrichment of these binding motifs in AHR binding sites suggested potential interaction with AHR. In fact, substantial AHR-SP1 interaction has been reported to be involved in the synergistic induction of CYP1A1, glutathione S-transferase, NQO1, aldehyde dehydrogenase-3, and UDP-Glucuronosyltransferase (Kobayashi et al., 1996). Like the AHR, hypoxia inducible factor belongs to the bHLH/PAS family and binds to DNA sequence (5’RCGTG3’) similar to the AHRE (Wenger et al., 2005). The similarity between the binding sequences of AHR and HIF was largely dependent on their common heterodimerization partner ARNT, which recognizes the 3’-half site GTG (Swanson et al., 1995). Interaction between E2F and AHR had tremendous implications in the regulation of cell cycle as AHR activation displaced co-regulatory protein such as p300 from E2F-dependent promoter. The displacement of p300 repressed the expression of E2F target genes such as Cdk2 and Cyclin E, leading to S-phase inhibition (Marlowe et al., 2004). NR2F1 (a.k.a. COUP-TF1) interacted physically with AHR and competes with AHR for AHRE binding, representing yet another negative regulatory pathway for AHR signalling (Klinge et al., 2000). AHR has also been reported to complex with EGR-1 in response to high glucose (Dabir et al., 2008). However, AHR-EGR-1 interaction in response to TCDD has not been investigated (Dabir et al., 2008). In summary, our transcription factor binding site analysis successfully identified many previously reported proteins that interacted with AHR and, in addition, many potential interacting proteins that have yet to be investigated in the context of AHR activation.

Despite the fact that the AHRE was significantly enriched in both our promoter-focused and genome-wide studies, not every AHR binding site contained an AHRE (Dere et al., 2011; Lo et al., 2011). In fact, the fraction of AHR binding sites containing at least one AHRE ranged from 50% to 68%, suggesting that there are alternate mechanisms to facilitate AHR-DNA interaction. At least two alternative response elements that might interact with AHR have been described – the AHRE-II (Boutros et al., 2004; Sogawa et al., 2004) and the non-consensus AHRE (Huang and Elferink, 2012).

The AHRE-II accounted for 5.8% and 50.2% of AHR-DNA interaction in AHR-bound regions lacking the core AHRE in mouse hepatic tissues treated with TCDD for 2 and 24 h respectively (Dere et al., 2011). The temporal discrepancy might be explained by the
differences in the size of the datasets (14,446 regions at 2 h and 974 regions at 24 h) (Dere et al., 2011). Alternatively, TCDD might induce the expression of an unknown protein, which in turn facilitates AHR-AHRE-II interaction at the later time point. In fact, it has been established that AHR does not bind AHRE-II directly but requires additional factors to bridge the AHR complex to DNA (Sogawa et al., 2004). The identity of the factor that mediated AHR-AHRE-II interaction remains unknown.

The non-consensus AHRE (NC-AHRE) was interesting in that it promoted AHR-DNA interaction at plasminogen activator inhibitor-1 (PAI-1) in the absence of its general heterodimerization partner, ARNT (Huang and Elferink, 2012). AHR-DNA interaction in the absence of ARNT might be an artifact due to the differences in antibody affinity and quality. I have also observed similar discrepancies between AHR and ARNT binding during my qPCR validation of AHR-bound regions in Chapter 3 (data not published). Such controversial findings have yet to be verified in other biological models and target genes. Systematic analysis of AHR and ARNT binding at other similar NC-AHRE sites should be conducted in AHR-deficient or ARNT-deficient models. Furthermore, AHR-DNA interaction through NC-AHRE in the absence of ARNT should be thoroughly investigated in human AHR and/or ARNT knockout cell lines (currently being engineered in our lab; Ahmed et al. submitted) to eliminate potential species-specific differences. If AHR can indeed be recruited to PAI-1 in the absence of ARNT, then AHR recruitment should not be affected in ARNT knockout models.

6.8 In vivo-in vitro differences in AHR signalling

In vivo-in vitro differences in TCDD response were well established between Hepa1c1c7 and mouse hepatic tissues. TCDD differentially regulated 619 genes over a period of 168 h in vivo, however only 67 of those 619 genes (~10%) were common between in vivo and in vitro models (Boverhof et al. 2005). Consistent with the gene expression data, our AHR binding site comparison between Hepa1c1c7 cells and mouse hepatic tissues also revealed a relatively low degree of overlap at <10% (62 common binding sites out of 747).
Pharmacokinetics might contribute to the discrepancy in TCDD response between in vivo and in vitro model. Whereas Hepa1c1c7 cells were exposed directly to 10 nM TCDD in culture (one compartment model), TCDD distribution in rodents is more complicated and might follow an eight compartmental model where TCDD is circulated and distributed into eight compartments such as blood, lung, kidney, skin, fat, spleen, liver, and the rest of the body (Emond et al., 2004). Upon oral ingestion, tissue concentration and TCDD uptake into the liver is membrane-limited and influenced by diffusion parameters (Emond et al., 2004). TCDD concentration in the liver peaked 24 h after oral administration (Emond et al., 2004), which correlated well with maximal AHR recruitment to CYP1A1 in vivo in our study (Figure 20). However, animals in our study were subjected to intraperitoneal injection. Orally administered TCDD would need to pass through the gastrointestinal tract and the hepatic portal vein before reaching the liver, while TCDD uptake into the liver might be more rapid through intraperitoneal injection as the majority of the TCDD would be taken up directly through the lining of the peritoneal cavity. Furthermore, differences between hepa1c1c-7 and mouse hepatic tissue might be due to the origin of the models. Hepa1c1c-7 cells were immortalized and derived from hepatoma tumours, whereas hepatic tissues were normal tissues. Furthermore, hepatocytes in vivo can participate in complex intercellular signalling pathways among other cell types such as stellate and kupffer cells, which might contribute to the in vivo-in vitro differences observed in our study.

6.9 ChIP-chip: AHR recruitment vs. gene expression in vivo

Expression microarray identified 133 differentially regulated genes in hepatic tissues treated with TCDD for 4 h. Of the 133 regulated genes, only 39 (29%) were associated with an AHR-bound region in our promoter-focused study. This low degree of correlation between binding events and gene regulation was also observed in our genome-wide study, in which

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1 The amount of TCDD deposited into the liver at 24 h is approximately 4.7% of the administrated dose, which is equivalent to approximately 0.52 nmol per 100 g tissue weight. This is relatively close to the 0.1 nmol TCDD that cells in culture are exposed to.
only 625 out of 1,896 (32%) TCDD regulated genes were AHR-bound. This apparent discrepancy between binding events and gene regulation can be explained by incomplete probe coverage, which would artificially under-estimate the number of AHR binding sites around regulated genes. In fact, our promoter-focused study failed to detect AHR recruitment to the Cyp1a1 AHRE cluster because that particular region was not tiled in the array. Furthermore, our stringent cutoff of FDR1 in our ChIP-chip experiments would result in a high rate of false negatives and might have excluded weak, albeit, positive AHR-bound regions. Lastly, the discrepancy might represent secondary responses to TCDD that occurred subsequent to AHR activation.

On the other hand, not all AHR binding sites were associated with gene regulation. There were many AHR-bound regions that were not associated with transcriptional changes in their neighbouring genes. It is likely that TCDD dependent induction was regulated by epigenetic mechanisms in addition to AHR interaction. In our present study, we investigated two epigenetic markers – H3K9Ac and H3K4Me2 – and their roles in AHR-mediated gene regulation. An increase in H3K9Ac and a decrease in H3K4Me2 were associated with AHR activation in hepa1c1c-7 cells; however, it remains unclear if similar epigenetic regulation might also occur in vivo. To this end, changes in H3K9Ac and H3K4Me2 were assessed in six genes associated with at least one AHR binding sites. Three of those genes (Cyp1b1, Nqo1, Nfe2l2) were TCDD responsive, while the other three (Gabarap, Erbb3, Ppara) were not responsive to TCDD. Increase in H3K9Ac was significantly associated with gene induction; however, contrary to in vitro data, decrease in H3K4Me2 was not an accurate predictor for AHR activation. Given the important regulatory role of histone modification, epigenetics might be another factor that contributes to in vivo-in vitro differences in gene expression profile between hepa1c1c7 and mouse hepatic tissues. Furthermore, the data demonstrated that epigenetic modification is an important aspect of AHR signalling and might be a good marker for AHR transcriptional activity in breast cancer.

6.10 ChIP-Seq: AHR binding sites in breast cancer

The comprehensiveness and the resolution of each ChIP-chip experiment relied heavily on the genomic coverage of the microarray platform. For example, Sartor et al. and Ahmed et
al. utilized the promoter-focused platform (GeneChIP ® mouse promoter 1.0R and GeneChIP ® human promoter 1.0R) (Ahmed et al., 2009; Sartor et al., 2009), whose coverage was limited to only 10 kb of 25,500 annotated genes (approximately 250,000,000 bases or <10% of the genome). While De Abrew et al. utilized a whole genome tiling array that covers the entire mouse genome, Dere et al. have shown that the array probes were distributed unevenly across the genome (Dere et al., 2011). This uneven distribution of probes resulted in gaps that overlapped with computationally determined AHRE cores, thereby missing potential biological relevant AHR binding sites (Dere et al., 2011). As discussed in the sections above, our understanding of genomic AHR signalling is limited by weaknesses such as incomplete probe coverage and hybridization biases. Massively parallel sequencing technology bypassed that problem by directly sequencing immunoprecipitated DNA without *a priori* information. DNA sequence can then be re-aligned to the reference genome for a high resolution mapping of genomic binding sites. Given the advantages of sequencing technology, we conducted ChIP-Seq to investigate genome-wide analysis of AHR and ARNT binding sites in MCF-7 cells in an attempt to better understand the role of AHR signalling in breast cancer.

Our ChIP-Seq identified 2,594 AHR and 1,352 ARNT binding sites in MCF-7 breast cancer cells at FDR1 and FDR5. Two different statistical cutoff levels were chosen to adjust for differences in antibody quality and to create two datasets of similar size for comparison. A lower than expected overlap (65%) between AHR and ARNT binding sites was observed. While there have been no reports of genomic ARNT binding sites upon TCDD treatment in the literature for reference, we expected a close to 90% overlap given the close relationship between the two heterodimeric partners. As discussed extensively in the previous sections, many factors might contribute to the discrepancy between the AHR and the ARNT datasets. These factors included a high rate of false negatives and inherent differences in antibody quality/affinity. Despite the discrepancy, our stringent cutoff and filtering method by comparing the AHR and the ARNT datasets allowed us to compile a list of high confidence AHR/ARNT binding sites that are not limited by probe coverage.
6.11 ChIP-Seq: Gene regulation by distally located AHR binding sites

The distribution of AHR/ARNT-bound regions suggested that AHR primarily regulates gene expression through binding to the proximal promoter area. However, our ChIP-Seq studies indicated that there were many remote AHR/ARNT-bound regions that might regulate gene expression distally as far as 100 kb away from their corresponding TSS (Figure 29). The precise mechanism by which distal AHR recruitment regulated gene expression was not investigated in our studies and remains unclear. Traditionally, *in vitro* studies with artificial reporter constructs were used to investigate the role of cis-regulatory elements in gene regulation limited to the proximal promoter area (usually less than 10 kb from the TSS). While transcription factor such as E2F binds exclusively to proximal promoter regions, many transcription factors such as p53, p63, FOXA protein, glucocorticord receptor, liver X receptor, retinoid X receptor, and estrogen receptor bind primarily to distal cis-acting enhancers (Cawley et al., 2004; Hurtado et al., 2011; Wederell et al., 2008; Yang et al., 2006). In fact, it has been estimated that only less than 10% of transcription factors have at least 50% binding sites located within 2.5 kb of TSS, suggesting that majority of transcription factors regulated gene expression distally (Farnham, 2009). Transcription factors have been postulated to regulate gene expression distally through at least three distinct mechanisms – looping, tracking, and linking (Figure 42) (Li et al., 2006a). The tracking model is when the distally bound transcription factor tracks or moves along the genome until it interacts with an active promoter (Li et al., 2006a). In the linking model, facilitator proteins extend from the distal enhancers to alter the transcriptional activity of adjacent promoters (Li et al., 2006a). The tracking model is unlikely as it would imply a series of intermediate AHR binding sites progressing from distal enhancers to proximal promoters, which we have not observed in our ChIP-Seq experiment (Figure 42). The linking model is also unlikely as it would imply that the entire genomic region spanning the distal enhancer and the proximal promoter would be crosslinked, immunoprecipitated, and sequenced in our assay (Figure 42). There is no evidence for such a large DNA-protein complex in our ChIP-Seq experiment. Hence, the looping model might be the best model to describe the mechanism of gene regulation by distal AHR binding sites.
The looping model brings two contact points - proximal promoter and distal enhancer - together to form an active transcriptional complex. The probability of the proximal promoter and distal enhancer to make contact depends on the flexibility of the chromatin structure, which is altered by histone modifications such as histone acetylation (Anderson et al., 2001). Histone modification, especially histone acetylation, has already been established to play critical roles in AHR-mediated gene regulation (Lo et al., 2011; Schnekenburger et al., 2007). Secondly, in the looping model, AHR bridges and makes direct contact at the distal enhancers and the proximal promoters. This is supported by the fact that our ChIP-Seq studies identified genes with corresponding AHR binding sites in both proximal promoter regions and distal enhancer regions (Figure 28). Lastly, a similar looping mechanism has been demonstrated for ligand-activated receptor such as the estrogen receptor alpha (discussed in section 6.4) using methods such as chromosome conformation capture assays (3C assays) and chromatin interaction analysis with paired end tags (ChIA-PET) (Deschenes et al., 2007; Li et al., 2010). Similar experiments have not been conducted for AHR. Elucidating the precise mechanism of distal gene regulation by AHR should be the priority for future AHR genomics studies.
Figure 42 Proposed models to explain gene regulation by distal binding sites.

The three proposed models are looping, linking and tracking. Only the looping mechanism would yield ChIP fragments that are compatible with our ChIP-Seq result. This figure was modified from (Li et al., 2006a).

6.12 Interaction between AHR and NRF2

Coordinated regulation of Phase I bioactivating and Phase II conjugating enzymes was mediated by crosstalk between AHR and NRF2. Hayes et al. suggested the bidirectional interplay between AHR and NRF2 is mediated through at least two mechanisms (Hayes et al., 2009). The first mechanism is reciprocal regulation between the two transcription factors. AHR activation induced NRF2 expression through several functional AHREs in the NRF2 promoter (Lo and Matthews, 2012; Miao et al., 2005). Similarly, NRF2 activation induced AHR expression through several functional AREs in the AHR promoter (Shin et al., 2007). Consistent with this proposed mechanism, TCDD was unable to induce NQO1 expression in NRF2-null mice, suggesting that TCDD induced NQO1 indirectly through NRF2 (Lin et al.,
2011; Ma et al., 2004). However, it remains unclear how AHR-dependent increases in NRF2 protein expression might circumvent the inhibitory effect conferred by KEAP1. The second mechanism involves physical interaction – be it direct or indirect – between AHR and NRF2. Physical interaction between AHR and NRF2 has not been examined and might influence the transactivation of both transcription factors. Physical interaction might occur through two scenarios. The first scenario is that the NRF2 binding site and AHR binding site are so close together in the genome that interaction is conceivable. This situation is best exemplified by the regulatory region of \( NQO1 \), which contains an ARE and an AHRE within 50 bp apart (Lin et al., 2011) (Figure 43 left). Our transcription factor binding site enrichment analysis in our ChIP-Seq study (Chapter 4) indicated that the ARE was significantly enriched within 50 bp of an AHRE in the majority of our AHR-bound regions (Table 8). The reciprocal was also true when we analysed the NRF2 ChIP-Seq dataset published by Chorley et al. (Chorley et al., 2012) . The AHRE was significantly enriched in NRF2 binding sites. Therefore, our AHR ChIP-Seq data and those by Chorley et al. together indicated a close relationship between AHRE and ARE in the genome (Chorley et al., 2012; Lo and Matthews, 2012).

The second scenario is that the NRF2 protein interacts directly with AHR protein in a large transcription complex. NRF2 activation in turn facilitates AHR recruitment to NRF2 binding sites. Consistent with this theory, our ChIP data demonstrated significant AHR and NRF2 recruitment to \( HMOX1 \) and \( NQO1 \) when both transcription factors were activated. AHR and NRF2 recruitment to \( NQO1 \) was expected since NQO1 contains both an AHR binding site and a NRF2 binding site (Lin et al., 2011) (Figure 43 left). On the other hand, \( HMOX1 \) lacked a consensus and functional AHRE. Furthermore, our ChIP-Seq and ChIP-chip experiments have shown that AHR did not bind to the regulatory region of \( HMOX1 \) upon TCDD treatment alone (Lo and Matthews, 2012). Yet significant AHR and NRF2 recruitment to \( HMOX1 \) was observed in cells treated with DIM+SFN or TCDD+SFN. Collectively, the data implied that simultaneous NRF2 activation by SFN influenced and facilitated the recruitment of AHR to \( HMOX1 \), likely through physical interaction and tethering mechanism (Figure 43 right). Our mRNA expression data in AHR knockdown cells also suggested that this potential interaction between AHR and NRF2 might synergistically enhance the transcription of NRF2 target genes.
Similar results were also observed by Saw et al. in a recent publication, where they reported synergistic induction of an ARE-dependent reporter gene in HepG2-C8 cells treated with DIM+SFN (Saw et al., 2011). Furthermore, drug-drug interaction between DIM and SFN leading to synergistic induction of NQO1, NRF2, HMOX1, SOD1, and UGT1A1 was demonstrated using the Loewe additivity model of drug-drug interaction (Saw et al., 2011).

![Figure 43 Dissecting the mechanism of AHR and NRF2 recruitment to NQO1 (left) and HMOX1 (right) under different treatment conditions.](image)

Unlike NQO1, HMOX1 lacks a functional AHRE. However, significant AHR recruitment is observed in cells treated with TCDD+SFN. The most likely explanation for the ectopic recruitment of AHR to HMOX1 is through physical interaction between NRF2 and AHR shown in the diagram.

Synergistic interaction between SFN and DIM is biologically relevant since oral consumption of cruciferous vegetables would result in simultaneous exposure to both compounds, leading to the activation of both AHR and NRF2. Although controversial, several epidemiological experiments have provided evidence for an inverse relationship between cruciferous vegetable consumption and breast cancer risk (Kim and Park, 2009). In a case-control study at hospitals in the western New York region, broccoli consumption in
premenopausal women was correlated with a modest decrease in breast cancer risk with an odd ratio of 0.6 (95% confidence interval 0.4 – 1.01) (Ambrosone et al., 2004). The finding was corroborated by a nationwide study in Sweden comparing the breast cancer risk between high and low cruciferous vegetable consumption in women (Terry et al., 2001). The high consumption arm had a significantly lower breast cancer risk with an odds ratio of 0.58 (95% confidence interval 0.42-0.79) compared to the low consumption arm. A meta-analysis from 17 studies concluded a significant reduction in breast cancer risk in high vegetable consumption group with relative risk of 0.75 (95% confidence interval 0.66-0.85) (Gandini et al., 2000). The precise mechanism by which cruciferous vegetable intake might reduce breast cancer risk is unknown but might be related to altered estrogen metabolism and induction of antioxidative enzymes through crosstalk between the AHR and NRF2 signalling pathways.

### 6.13 Interaction between ER\(\alpha\) and NRF2

Interaction between ER\(\alpha\) and NRF2 was first reported by Montano et al. who demonstrated significant increase in NQO1 expression in MCF-7 exposed to 4-hydroxytamoxifen, an ER partial agonist (Montano and Katzenellenbogen, 1997). Initially, it was believed that ER\(\alpha\) negatively regulated NQO1 by directly interacting with the ARE in the promoter region (Montano et al., 1998). Subsequent studies have suggested that rather than competing with NRF2 for interaction with ARE, ER\(\alpha\) might inhibit NRF2 transactivation through physical interaction (Ansell et al., 2005) (Figure 44). However, it remains unclear which mechanism best describes ER\(\alpha\) dependent repression of NRF2 activity. Our ChIP experiments demonstrated significant ER\(\alpha\) recruitment to NRF2 binding sites at **HMOX1** and **NQO1** only in cells co-treated with E2+SFN or DIM+SFN. Moreover, ER\(\alpha\) recruitment did not affect the recruitment of NRF2. Collectively, our ChIP data and those by Ansell et al. do not support competition between NRF2 and ER\(\alpha\) for interaction with ARE as the basis of NRF2 repression (Ansell et al., 2005). ER\(\alpha\)-dependent repression of NRF2 activity might be mediated through protein-protein interaction in the same transcriptional complex, which can be examined using sequential ChIP.
Figure 44 Potential mechanism of ERα recruitment to NRF2 target genes.

While Montano et al. have suggested competition between ERα and NRF2 as a mechanism of ERα mediated repression of NRF2 target gene expression, our data and those of Ansell et al. suggested physical interaction between ERα and NRF2 at the genomic level (Ansell et al., 2005; Montano et al., 1998).

Crosstalk between NRF2 and ERα has tremendous biological implication, especially in oxidative and endocrine-related diseases such as breast cancer. In Chapter 5, I have demonstrated that NQO1, an NRF2 target gene, was significantly repressed by ERα. NQO1 is an oxidoreductase primarily responsible for the reduction of reactive estrogen quinones (Gaikwad et al., 2007). Recent studies have shown that tamoxifen treatment can enhance NQO1 expression through estrogen receptors, ultimately resulting to a decrease in DNA damage and mammary tumorigenesis (Krishnamurthy et al., 2012; Montano et al., 1998; Sripathy et al., 2008). Similarly, Yao et al. observed an increase in overall tumor weight when shRNA NQO1 knockout MCF-7 cells were xenografted in mice (Yao et al., 2010). Hence, ERα-mediated repression of NQO1 might represent one of the key molecular events predisposing breast tissue to tumorigenesis.

Interestingly, the two different estrogen receptor subtypes might affect NRF2 transactivation and NQO1 expression differently. Montano et al. have demonstrated that while both ERα and ERβ can regulate the expression of NQO1, ERβ was the stronger ER subtype at mediating 4-hydroxytamoxifen dependent induction of NQO1 (Montano et al.,...
1998). In other words, enhanced expression or transactivation of ERβ might increase NQO1 expression and might ultimately decrease critical mutation leading to breast cancer. This was in agreement with the fact that positive breast cancer prognosis was correlated with the balance between ERβ and ERα expression (Matthews and Gustafsson, 2003). For example, normal breast tissue exhibited higher ERβ-to-ERα ratio compared to breast tumors (Lazennec et al., 2001; Roger et al., 2001). While some studies have shown that ERβ might prevent breast cancer progression by altering cell cycle regulation and cell proliferation (Lazennec et al., 2001; Paruthiyil et al., 2011; Sotoca et al., 2008), it might also prevent E2-related DNA damage and initiation of genotoxic events by differentially up-regulating protective enzymes such as NQO1 (Krishnamurthy et al., 2012; Montano et al., 1998; Sripathy et al., 2008).

Furthermore, while ERα interacted physically with NRF2 to repress NRF2 activity through its AF-1 and DNA binding domain, it remains unclear how or if ERβ interacts with NRF2 (Montano et al., 1998). While ERα and ERβ exhibited close to 97% sequence homology in their DNA binding domains, their AF-1 domains were substantially different with <20% sequence homology (Lo and Matthews, 2010). Moreover, the AF-1 domain of ERβ has been reported to be weaker compared to that of ERα (Cowley and Parker, 1999; McInerney and Katzenellenbogen, 1996). Interaction between ERβ and NRF2 should be investigated in the future given its importance in the context of breast cancer.

6.14 HMOX1 and breast cancer

Free floating hemes in the body participate in the fenton reaction to generate free radicals. The physiological role of HMOX1 is to convert free floating pro-oxidative heme into carbon monoxide, ferrous ion Fe\(^{2+}\), and biliverdin (Gozzelino et al., 2010). The resulting Fe\(^{2+}\) is then oxidized into Fe\(^{3+}\) by ferritin H and stored safely in ferritin complexes, neutralizing the oxidative effect of Fe (Gozzelino et al., 2010). The entire process reduces the pro-oxidative hemes into neutralized and ferritin-bound Fe, hence HMOX1 has been considered to be an antioxidative gene and cellular protective enzyme. In this thesis, we have demonstrated
crosstalk between AHR and NRF2 leading to a significant increase in HMOX1 expression that was approximately >80 fold higher in TCDD+SFN and DIM+SFN treated cells compared to vehicle control in MCF-7 breast cancer cells. Similar results were observed in breast cancer cells such as T-47D and ZR-75, but not MDA-MB-231 (data not shown). However, the biological implication of this robust induction in breast cancer was unexplored in our study. Moreover, HMOX1’s role in tumorigenesis remains controversial.

Whereas NQO1 is an established antitumorigenic and detoxification enzyme in breast cancer (Lu et al., 2008; Yao et al., 2010), HMOX1 has been reported to be protumorigenic and antitumorigenic depending on the cellular context and experimental conditions (Jozkowicz et al., 2007). Elevated HMOX1 expression was associated with a wide range of cancers including lymphosarcoma, adenocarcinoma, hepatoma, glioblastoma, melanoma, prostate cancers, Kaposi sarcoma, squamous carcinoma, pancreatic cancer, and brain tumors (Summarized in (Jozkowicz et al., 2007)). HMOX1 induction was associated with faster tumor growth in pancreatic cancer, angioma, and melanoma, while HMOX1 inhibition resulted in tumor suppression. The underlying mechanism by which HMOX1 induced tissue-specific cell proliferation was unclear and might involved the down-regulation of cell cycle signal molecules such as p21 and B-cell translocation gene-2 (BTG2) and the up-regulation of mitogenic factors such as epidermal growth factors (EGF) (Was et al., 2006). p21 is a cyclin-dependent kinase inhibitor whereas BTG2 is a tumor suppressor. Both p21 and BTG2 interacted with and inhibited CdK2-dependent phosphorylation of Rb, regulating G1/S transition. The down-regulation of p21 and BTG2 observed in HMOX1 over-expressed cells likely contributed to HMOX1’s pro-proliferative effect. EGF, on the other hand, is a well-established mitogen that activates EGF receptors to initiate a cascade of protein kinase activities, ultimately leading to cell proliferation and differentiation (Zaczek et al., 2005).

HMOX1 over-expression was associated with clinical stage III breast cancer (Folgueira et al., 2006). The staging system graded different breast cancer cases into three clinical stages based on the size and the nature of the primary tumor, the severity of the regional lymph nodes affected, and the degree of metastasis (Singletary et al., 2002). As outlined above, most studies pointed to HMOX1 as a pro-tumorigenic enzyme. However, one study examined the effect of HMOX1 over-expression in rat and human breast cancer
cells and concluded that HMOX1 might be anti-proliferative in breast cancer (Hill et al., 2005). HMOX1 dependent inhibition of cell proliferation was mediated by an increase in bilirubin production, which led to cell proliferation inhibition. Moreover, HMOX1 over-expression was associated with cell cycle arrest in the G0/G1 phase and an increase in apoptosis in MCF-7 cells. Hence, the effect of HMOX1 on cancer growth might be tissue specific. Our data in MCF-7 cells demonstrated that ERα knockdown coupled with DIM+SFN treatment resulted in enhanced HMOX1 induction, which might be desirable in the context of breast cancer. Collectively, these observations supported the hypothesis that breast cancer patients might benefit more from fulvestrant (ER degrader) treatment coupled with a high dietary intake of cruciferous vegetables. However, unlike NQO1, the effect of HMOX1 on breast cancer development and progression is still poorly understood. HMOX1’s tissue-specific, anti-proliferative effects in breast cancer should be verified and further investigated in the future.
Chapter 7 Limitations, Future Directions, and Summary

7.1 Limitations

*Hybridization based array vs. Sequencing based technology*

ERα and AHR binding sites in Chapter 2 and 3 were determined using ChIP-chip, a hybridization based method. Some of the limitations associated with hybridization arrays in our studies included hybridization bias, probe coverage, low signal-to-noise ratio, saturation, and low sensitivity. In order to generate enough input material for hybridization, we amplified our ChIPed DNA using a whole genome amplification kit. The amplification process might also introduce biases to our samples. Furthermore, both studies in Chapter 2 and 3 were performed using promoter-focus arrays, which limited the coverage of our binding site analysis to regions approximately 10 kb around annotated TSS. Therefore, we might have missed biologically relevant ERα and AHR binding sites distal from TSS.

Sequencing based methods circumvented most of the limitations associated with hybridization based methods. Hence, AHR binding sites were determined using ChIP-Seq in Chapter 4. Our sequencing based studies, however, were associated with their own weaknesses, including heavy bioinformatics analysis and stringent sample submission requirement.

*Cell culture models, primary mammary epithelial cells, and normal mammary epithelial cells*

We have used immortalized breast cancer cells as a model for breast cancer. Although breast cancer cell lines such as MCF-7 and T-47D cells are responsive to estrogen and have traditionally been used to examine estrogen signalling in breast cancer, they might not accurately represent the biology of estrogen in normal breast epithelial cells or even breast cancer cells. Normal breast epithelial cells have been reported to express significantly lower levels of ERα compared to their cancerous counterparts (Lofgren et al., 2006). Commercially available primary mammary epithelial cells that were immortalized by telomerase reverse transcriptase (TERT) did not express ERα at all (in house data and ATCC datasheet). The
different levels of ER$\alpha$ expression in the different biological models represented a limitation in our studies and a challenge in breast cancer research in general.

Specificity/Sensitivity of Antibodies

The success of the ChIP assays in our studies relied heavily on the quality of the antibody. Although ARNT is the general heterodimerization partner of AHR, the binding site overlap between the two proteins was lower than expected in Chapter 4. Differences in antibody affinity and specificity might explain this low degree of overlap between AHR and ARNT. This issue can be addressed by repeating the experiments with antibodies from a different source or antibodies that recognize a different epitope. For example, ARNT binding sites were verified using an antibody (Novus Biologicals) that was different from the one used in the ChIP-Seq experiments (Santa Cruz).

7.2 Future Directions

Gene regulation by distal AHR binding sites

Our recent ChIP-Seq study presented in Chapter 4 revealed several TCDD responsive genes with corresponding AHR binding sites greater than 10 kb away (Lo and Matthews, 2012). How do AHR binding sites at distal enhancers communicate with the promoter to regulate gene expression? Studies from nuclear receptors such as ER$\alpha$ and GR suggested that distal transcription factor binding sites might regulate gene expression by changing chromatin conformation in a mechanism called DNA looping (Li et al., 2010; Reddy et al., 2009). To elucidate the mechanism of gene regulation by distal AHR binding sites, chromatin interaction analysis using pair end tags (ChIA-PET) (Li et al., 2010) and chromosome conformation capture assays (3C assays) (Deschenes et al., 2007) should be conducted in cells exposed to TCDD. Given potential ligand specific responses in AHR activation (Pansoy et al., 2010), it might also be interesting to examine DNA looping in cells exposed to other AHR ligands such as DIM.

Genomic binding pattern of AHR in untreated cells
One of the weaknesses of our ChIP-Seq study was that we did not examine the genomic binding pattern of AHR in unstimulated cells. AHR shuttles between the cytoplasm and the nucleus (Ikuta et al., 2000) and has been shown to interact with DNA in the absence of ligand activation (Ahmed et al., 2009; Sartor et al., 2009). Interestingly, Sartor et al. also reported that a large percentage of AHR bound genes in untreated cells was not bound by AHR in ligand-treated cells, suggesting differential AHR binding pattern in treated and untreated cells (Sartor et al., 2009). High-throughput determination of AHR binding sites in untreated cells should be conducted to assess the physiological role of AHR in the absence of ligand activation. One potential confounding variable, which should be controlled for in future experiments investigating the basal activity of AHR, might be the presence of tryptophan metabolites such as FICZ in the culture medium. FICZ has been reported to be a potent AHR ligands generated from the breakdown of tryptophan (Wei et al., 2000; Wincent et al., 2012).

Crosstalk among AHR, ERα, and NRF2

ChIP assays demonstrated AHR and ERα recruitment to the NRF2 binding sites of NQO1 and HMOX1 only in cells treated with DIM+SFN, suggesting that NRF2 activation might influence the genome-wide binding pattern of AHR and ERα. Given the increasing accessibility of massively parallel sequencing, future experiments should examine changes in the genome-wide binding pattern of AHR and ERα in cells treated with DIM+SFN using ChIP-Seq. Furthermore, it remains unclear from our studies how AHR and ERα might interact with NRF2 at NQO1 and HMOX1. Physical interaction between ERα and NRF2 has been reported (Ansell et al., 2005). However, AHR and NRF2 interaction has not been investigated. Do AHR and ERα interact physically with NRF2 or does NRF2 recruitment open up the chromatin and make the adjacent genomic regions more accessible to AHR and ERα? The precise mechanism of interaction should be examined by utilizing functional mutants of AHR and ERα.

Does the non-additive induction of NQO1 and HMOX1 by DIM+SFN correlate with decreased metabolite formation or decreased DNA damage?
Our study presented in Chapter 5 demonstrated that DIM+SFN resulted in enhanced induction of two NRF2 target genes, *NQO1* and *HMOX1*. This finding was corroborated by a recent report by Saw et al., describing the synergistic induction of several NRF2 target genes in cells co-treated with DIM and isothiocyanate (Saw et al., 2011). Does co-treatment with DIM+SFN confer protection against the formation of genotoxic metabolites such as estrogen quinones in breast cancer? NQO1 is the primary oxidoreductase that reduces and detoxifies the reactive estrogen quinones (Gaikwad et al., 2007; Lu et al., 2008). Furthermore, Lu et al. have definitively demonstrated that an increase in NQO1 expression and enzymatic activity is correlated with a decrease in the formation of catechol estrogens and depurinating adducts (Lu et al., 2008). Interestingly, Lu et al. conducted their experiments in non-tumorigenic MCF-10F cells which do not express ERα (Lu et al., 2008). Given that ERα has an inhibitory effect on NRF2 activity (Montano et al., 1998; Montano and Katzenellenbogen, 1997), how might ERα expression in MCF-7 influence the protective effect conferred by NQO1? Future studies should be conducted in MCF-7 to assess whether the induction of NQO1 expression by DIM+SFN will enhance the detoxification of estrogen metabolites and decrease the formation of bulky DNA adducts with the use of liquid chromatography coupled with mass spectrometry.

*How does HMOX1 expression affect breast cancer progression and development?*

HMOX1 deficient animals are susceptible to cardiovascular injury (Immenschuh and Schroder, 2006). However, their susceptibility to breast or other forms of cancer has not been thoroughly studied. One study reported earlier onsets of skin lesions and squamous cell carcinoma in *Hmox1*−/− and *Hmox1*+/- mice applied with topical 7,12 dimethylbenz[a]anthracene (DMBA) (Was et al., 2011). *Hmox1*−/− fibroblasts exhibited enhanced DMBA-induced p21, cyclin-D1, and cyclin-D2 expression compared to their wild-type and heterozygous counterparts in culture, suggesting potential changes in cell cycle regulation (Was et al., 2011). However, similar studies have not been conducted with respect to breast cancer. DMBA is commonly used to induce mammary carcinoma through subcutaneous injection directly beneath the mammary glands. It would be interesting to examine breast cancer development/progression in those HMOX1−/− animals to dissect the biological implications of HMOX1 in breast cancer.
7.3 Summary

There are two co-existing molecular pathways that contribute to hormonal dependent breast cancer. Estrogen is a mitogen and can induce cell proliferation and tumor progression. At the same time, estrogen can be metabolized to genotoxic metabolites, which cause mutations in the genome to initiate tumor formation. The formation and the elimination of those metabolites are closely regulated by the expression of Phase I and Phase II enzymes. This thesis investigated the genomic action of ERα, AHR, and NRF2, which might play important roles in breast cancer by modulating the Phase I and Phase II gene expression.

1. ERα is involved in the regulation of Phase I bioactivating enzymes such as CYP2B6 in addition to CYP1A1, CYP1B1, and CYP2A6 in ERα positive breast cancer cells.
   • Up-regulation of Phase I bioactivating enzymes by ERα might enhance the generation of genotoxic metabolites in the development/progression of breast cancer

2. Genome-wide analysis of AHR binding sites indicates that AHR is a key regulator of CYP1A1, CYP1B1, NQO1, and NRF2 in hepatic tissues and in breast cancer cells. AHR can regulate gene expression through binding to distal enhancers and proximal promoters. Furthermore, AHR-mediated gene regulation involves epigenetic control such as histone acetylation.
   • Up-regulation of Phase I bioactivating enzymes by AHR might enhance the generation of genotoxic metabolites in the development/progression of breast cancer

3. AHR enhances, whereas ERα represses NRF2 target genes through altered p300 recruitment and histone acetylation.
   • Coordinated up-regulation of Phase II conjugating enzymes by AHR and NRF2 might protect breast cancer cells from genotoxic metabolites. Conversely, ERα-mediated repression of Phase II conjugating enzymes might enhance the generation of genotoxic metabolites.
References


Bonnese, C., Eggleston, I.M., and Hayes, J.D. (2001). Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer research 61, 6120-6130.


De Abrew, K.N., Phadnis, A.S., Crawford, R.B., Kaminski, N.E., and Thomas, R.S. (2011). Regulation of Bach2 by the aryl hydrocarbon receptor as a mechanism for suppression of B-cell differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicology and applied pharmacology 252, 150-158.


metabolizing enzymes and antioxidant genes. Toxicological sciences : an official journal of the Society of Toxicology 59, 169-177.


Korkalainen, M., Tuomisto, J., and Pohjanvirta, R. (2004). Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) of aryl hydrocarbon receptor
repressor in a TCDD-sensitive and a TCDD-resistant rat strain. Biochemical and biophysical research communications 315, 123-131.


Ma, Q., Kinneer, K., Bi, Y., Chan, J.Y., and Kan, Y.W. (2004). Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. The Biochemical journal 377, 205-213.


Peterson, T.J., Karmakar, S., Pace, M.C., Gao, T., and Smith, C.L. (2007). The silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor is required for full estrogen receptor alpha transcriptional activity. Molecular and cellular biology 27, 5933-5948.


Varlakhanova, N., Snyder, C., Jose, S., Hahn, J.B., and Privalsky, M.L. (2010). Estrogen receptors recruit SMRT and N-CoR corepressors through newly recognized contacts
between the corepressor N terminus and the receptor DNA binding domain. Molecular and cellular biology 30, 1434-1445.


